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FACULDADE DE MEDICINA VETERINÁRIA

MUCOSAL AND PHYSIOLOGICAL RESPONSES OF ATLANTIC SALMON (*Salmo salar*) IN BRACKISH WATER RAS FOLLOWING PERACETIC ACID-BASED DISINFECTION

JOÃO VASCO DE CARVALHO OSÓRIO

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Doctor Carlo Lazado
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To my grandfather José

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Atlantic salmon counteracts oxidant-induced oxidative stress through activation of mucosal and circulating antioxidant defences.

Manuscript ID: CBPB-D-20-00342

Resumo

O ácido paracético (PAA), um desinfetante com fortes propriedades oxidantes, é eficaz contra diversos microrganismos a baixas concentrações, requer um curto tempo de contacto e degrada-se rapidamente em resíduos inócuos, sendo, portanto, considerado uma alternativa promissora para a desinfeção de rotina em aquacultura. No entanto, é necessário um extenso conhecimento relativo aos impactos do PAA na saúde dos peixes para garantir a sua utilização segura.

Este estudo documentou as consequências fisiológicas da exposição periódica ao PAA em Salmão do Atlântico (*Salmo salar*) na fase "post-smolt", produzido num sistema de recirculação em aquacultura (RAS) de água salobra. Os peixes foram expostos ao PAA a uma concentração de 1 mg/L a cada 3 dias durante 6 semanas. Foram realizadas três recolhas extensivas de tecidos (antes da exposição, e aos dias 22 e 45 de exposição periódica). Além disso, foi realizado um desafio de stress antes do início de exposição e no dia 45 de exposição para avaliar os efeitos da exposição periódica na resposta a um estímulo secundário de stress. Durante o estudo não foi observado nenhum padrão óbvio na evolução dos parâmetros plasmáticos de stress, excetuando os níveis de glucose, que desceram significativamente ao longo do tempo. O stress oxidativo foi induzido provavelmente pela exposição periódica ao oxidante, tal como indicado pelo aumento nos níveis de antioxidantes plasmáticos. A expressão dos genes que codificam antioxidantes, citoquinas, proteínas de choque térmico e mucinas revelou que existe um padrão tecidular específico em resposta ao PAA: foi registado um padrão de inibição nas brânquias e na roseta olfatória, um padrão de indução na pele, enquanto no fígado não foram registadas alterações. A exposição ao PAA provocou alterações histológicas nas brânquias, pele e roseta olfatória, sendo as alterações predominantemente observadas nas brânquias, onde as alterações mais comuns foram casos de edema epitelial, hipertrofia, hiperplasia e "lamelar clubbing". A exposição periódica ao PAA não afetou a capacidade do salmão para estabelecer uma resposta fisiológica eficiente na presença de um estímulo indutor de stress.

De forma geral, este estudo demonstrou que a exposição periódica ao PAA constituiu um estímulo stressante para o qual os peixes foram capazes de apresentar respostas adaptativas, tanto a nível sistémico como nas mucosas. Além disso, a exposição ao PAA promoveu a manutenção da qualidade microbiológica da água e não afetou a performance do biofiltro. As respostas observadas neste protocolo de desinfeção destacam o potencial do PAA como um desinfetante de rotina na produção de salmão em RAS.

Palavras-chave: *Salmo salar*; desinfeção; saúde dos peixes; stress oxidativo; sistema de recirculação em aquacultura.

Abstract

Peracetic acid (PAA), a strong oxidative disinfectant, is effective against several microorganisms at low concentrations, requires short contact time and degrades rapidly into innocuous residues, thus considered a promising option for routine disinfection in aquaculture production. However, comprehensive knowledge of the impacts of the oxidant PAA on fish health is required for its safe application.

This study documented the physiological impacts of periodic PAA exposure in Atlantic salmon (*Salmo salar*) post-smolts reared in brackish water recirculating aquaculture system. Salmon were exposed to PAA at a concentration of 1 mg/L every 3 days over 6 weeks. Three extensive tissue samplings were conducted (before exposure, 22 and 45 days of periodic PAA exposure). In addition, a stress test was performed before exposure and 45 days post-exposure to assess the effects of periodic exposure during a secondary stress encounter. There was no clear pattern on the changes in plasma stress parameters throughout the exposure trial, except with the glucose level, which significantly decreased over time. Oxidative stress was likely triggered by periodic oxidant exposure, as indicated by the documented significant increase in plasma antioxidants. PAA-induced expression of genes encoding for antioxidants, cytokines, heat shock proteins and mucins demonstrated a tissue-specific pattern: downregulation was observed in the gills and olfactory rosette, upregulation occurred in the skin, and no changes in the liver. Periodic oxidant exposure resulted in histological changes in key mucosal organs (olfactory rosette, skin and gills); pathological alterations were predominant in the gills where cases of epithelial lifting, hypertrophy, hyperplasia and lamellar clubbing were the most commonly identified. Lastly, periodic oxidant exposure did not alter the ability of salmon to mount robust physiological stress responses to a secondary stressor.

Collectively, the present study demonstrated that periodic PAA exposure constituted an environmental stressor for which salmon were capable of mounting adaptive responses, both at the systemic and mucosal levels. In addition, periodic PAA exposure promoted the maintenance of stable microbiological water quality and did not affect the biofilter performance. The consequences of this disinfection protocol underscored the potential of PAA as a routine oxidant-based disinfection in salmon RAS production.

Keywords: *Salmo salar*; disinfection; fish health; oxidative stress; recirculating aquaculture system.

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List of Abbreviations and Symbols

© - Copyright

® - Registered brand

‰ - Permillage

AB-PAS - Periodic acid schiff-alcian blue

actb - β-actin

ACTH - Adrenocorticotrophic-hormone

am - Ante meridiem

ANOVA - Analysis of variance

arp - Acidic ribosomal protein

AV - Analysis Value

BQV - Bactiquant® Values

BV - Blank Value

ca - Circa

cat - Catalase

cDNA - Complementary deoxyribonucleic acid

cu/znsod - Copper/zinc superoxide dismutase

CV - Combined Blank Value

ef1α - Elongation factor alpha-1

ELISA - Enzyme-linked Immunosorbent Assay

EtOH - Ethanol

FAO - Food and Agriculture Organisation of the United Nations

GOD - Glucose oxidase

gpx - Glutathione peroxidase

gr - Glutathione reductase

gsta - Glutathione S-transferase

H_2O_2 - Hydrogen peroxide

HPI - Hypothalamic-pituitary-interrenal

HRP - Horseradish peroxidase

hsp70 - Heat-shock protein 70

hsp90 - Heat-shock protein 90

hsp - Heat-shock protein

il1β - Interleukin 1β

kDa - Kilo daltons

LC50 - Median lethal concentraion

MALT - Mucosal-associated lymphoid tissues

MBBR - Moving bed biofilter reactor

mnsod - *Manganese superoxide dismutase*

MS-222 - Tricaine methanesulfonate

muc2 - *Mucin2-like*

MUC5AC - Mucin 5AC

muc5ac - *Mucin5ac-like*

MUC5B - Mucin 5B

muc5b - *Mucin5b-like*

Na-/K+-ATPase - Sodium-potassium adenosine triphosphatase

NALT - Nasal-associated lymphoid tissues

NCRA - Nofima Centre for Recirculation in Aquaculture

NOEC - No observed effect concentration

O_2 - Oxygen

OD - Optical density

OSNs - Olfactory sensory neurons

p - p-value

PAA - Peracetic acid

PBS - Phosphate buffered saline

pH - Negative logarithm of the hydrogen ion concentration

pm - Post meridiem

PO_2 - Partial pressure of oxygen

qPCR - Quantitative polymerase chain reaction

RAS - Recirculating aquaculture system

RNA - Ribonucleic acid

ROS - Reactive oxygen species

rpm - Rotations per minute

RT-qPCR - Real-time quantitative polymerase chain reaction

SE - Standard error

TAC - Total antioxidant capacity

TM - Trademark

v/v - Volume per volume

α-MSH - Alpha-Melanocyte-stimulating hormone

β - Beta

I. Curricular Externship

As a part of the final year of the integrated Master's degree programme in Veterinary Medicine, a 5-month externship at Nofima, The Norwegian Institute of Food, Fisheries and Aquaculture Research took place between 2nd of September 2019 and 28th of February 2020, with the objective of participating in a trial aiming to document the impacts of peracetic acid disinfection on water quality, biofilter performance, and the health and welfare of Atlantic salmon reared in brackish water RAS. The externship was divided in two parts, with the first part taking place at the Nofima Centre for Recirculation in Aquaculture (NCRA) located in Sunndalsøra, Norway, and the second one at the Nofima Fish Health Laboratory located in Ås, Norway.

1.1 Nofima - The Norwegian Institute of Food, Fisheries and Aquaculture Research

Nofima is one of Europe's largest institutes for applied research within the fields of fisheries, aquaculture and food research. The institute was established on 1st of January 2008. It is organized in three divisions: Food science, Aquaculture and Seafood. It has 394 employees of whom 201 are R&D personnel. The main office is situated in Tromsø, with satellite offices in Ås, Bergen, Stavanger, Sunndalsøra and Alta. The Aquaculture Division has 4 departments namely Breeding and genetics, Fish health, Nutrition and feed technology and Production biology, as well as 3 aquaculture research stations (Centre for Marine Aquaculture, Tromsø Aquaculture Research Station and Research Station for Sustainable Aquaculture).

1.2 Nofima Centre for Recirculation in Aquaculture, Sunndalsøra

During his 2-month stay at the NCRA, the student was actively involved in performing the experimental trial that originated the results presented in the current study, under the joint supervision of Dr. Kevin Stiller and Dr. Carlo C. Lazado.

Figure 1 – The Nofima Centre for Recirculation Aquaculture (NCRA), located in Sunndalsøra, Norway. Source: https://nofima.no/en/

The student was entrusted with routine fish husbandry tasks that included feeding, daily visual inspection, maintenance of the RAS, collection of water samples, blood sampling and others. Once a week, water samples and media from the biofilter were collected and analysed to monitor water quality of the tanks and biofilter performance over the duration of the trial. The student also participated in 3 extensive samplings that provided him with practical experience in handling and collection of fish samples for analysis.

Besides collaborating in the execution of the present study, the student was given the opportunity to follow and assist the technical staff with technical maintenance procedures, routine husbandry, and collection of samples for other experimental trials that were being conducted in the station at the time.

1.3 Nofima Fish Health Laboratory, Ås

In the first week of November, following the termination of the experimental trial, the student moved to Nofima's Fish Health Laboratory in Ås, where he stayed until the end of the externship to process and analyse the samples collected from the fish trial at NCRA. The student was under the supervision of Dr Carlo C. Lazado, who gave him hands-on training on multiple laboratory techniques. Namely, the student learned enzyme-linked immunosorbent assay (ELISA), descriptive and quantitative histology, ribonucleic acid (RNA) isolation, complementary deoxyribonucleic acid (cDNA) synthesis, and real-time quantitative polymerase chain reaction (RT-qPCR), which he employed in the laboratory analyses described in this thesis. Afterwards, the obtained data were managed, and statistics were performed using R studio (version 1.2.5019) prior to interpretation of the trial results.

Figure 2 - Nofima in Ås, Norway, where the Fish Health Laboratory is located. Source: https://nofima.no/en/

II. Introduction

According to the Food and Agriculture Organisation of the United Nations (FAO), approximately 75% of the marine fish stocks are either fully exploited, overexploited, or in some cases even collapsed (FAO 2006). More recent data reveal that the percentage of marine fish stocks captured within sustainable levels has decreased from 90% in 1974 to 66.9 % in 2015 (FAO 2018). Since the 1980s, the figures for wild captures have been fairly stable, with aquaculture production surging as the main responsible for the staggering continued growth that has been witnessed in the supply of fish for human consumption. From 1961 to 2016, the average annual population growth (1.6%) was surpassed by the growth of the global food fish consumption (3.2%). In fact, food fish consumption increased from 9.0 kg per capita in 1961 to 20.2 kg in 2015, at an annual rate of approximately 1.5 %. While the global consumption of fish protein is expected to continuously grow in the future, capture fisheries alone cannot supply the increasing market necessities. Thus, future global demands in terms of fish consumption can only be met through effective aquaculture production (FAO 2018).

Culture of aquatic organisms is an ancient practice of humankind, with oyster production for instance, being traced back to ancient Rome and Gaul (Cole et al. 2009). Following the dramatic increase observed in the aquaculture sector during the second half of the 20th century, the annual growth rates for aquaculture production have since decreased to a modest 5.8% during the period of 2001-2016. Nonetheless, the contribution of aquaculture to the global production of food fish continues to rise, reaching a percentage of 46.8 in 2016, and continues to grow more rapidly than any other major food supply industry (FAO 2018).

Along with the necessity of maximizing aquaculture production to guarantee food security to the global population, aquaculture has recently become focus of public concerns regarding sustainability and animal welfare (Cole et al. 2009; Martins et al. 2010; Lindland et al. 2019). Therefore, efforts have been made towards developing novel technologies and solutions that maximize production thus allowing a long term growth of the aquaculture sector, while minimizing the possible environmental impacts and assuring the welfare of farmed fish, which in turn will provide aquaculturists with high quality products and contribute to the general public acceptance of the produced food fish (Verbeke et al. 2007; Martins et al. 2010; Dalsgaard et al. 2013).

III. Monography

1 Literature Review

1.1 The Atlantic salmon – geographical distribution and biological aspects

The Atlantic salmon (*Salmo salar*) is a teleost fish from the Salmonidae family. Wild populations historically occur alongside both west and east coasts of the North Atlantic Ocean. In the west side of the North Atlantic, salmon can range roughly from the Connecticut River to Ungava Bay in the North, whereas in the Northeast Atlantic salmon occur from North of Portugal to rivers flowing into the Barents and White Sea areas (MacCrimmon and Gots 1979). Atlantic salmon is a well-known anadromous species, hatching in streams and rivers but migrating to sea to grow, and therefore migratory movements are an essential part of its life history. As juvenile fish reach a certain stage of development, they leave their freshwater habitat, usually in spring, and start a downwards migration trough the river and estuaries to reach the ocean, where they look for deep water feeding grounds to grow and mature. Prior to and during this migration, juvenile fish undergo the process of "smoltification", an adaptive specialization that makes downstream migration and life at sea possible. During smoltification, fish become silvery and streamlined, lose their territoriality and positive rheotactic behaviour, adopt downstream orientation and schooling behaviour, and experience a series of physiological adaptations such as increased salinity tolerance, scope for growth, and metabolic rate. While these physiological changes are regulated by photoperiod and temperature, seaward migration is likely elicited by changes in temperature and water flow (McCormick et al. 1998; Klemetsen et al. 2003).

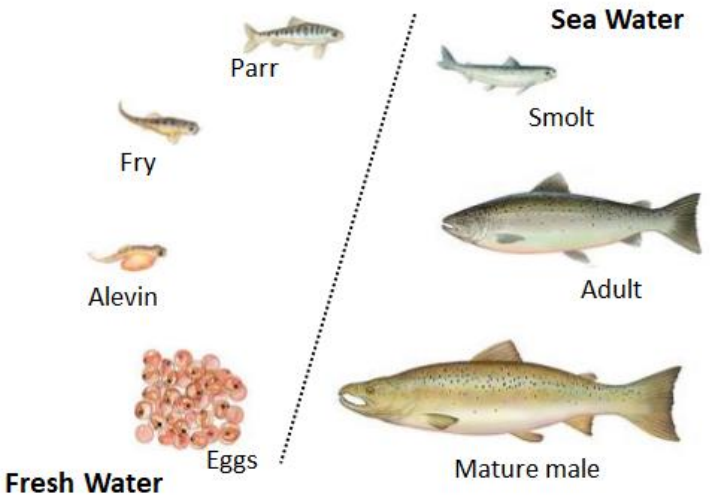

Figure 3 - The illustration represents the entire life cycle of Salmo salar. Adapted from Mirimachi Salmon Association, https://miramichisalmon.ca/education/atlantic-salmon/

Salmon can stay up to 4 years at sea feeding on pelagic species to grow, and when maturation starts, fish will stop feeding and return to their native rivers to spawn, normally form

October to January. As opposed to other salmonids, Atlantic salmon is iteroparous, meaning some individuals may survive spawning and return to the sea. Eggs are expelled and fertilized in upstream gravel nests and hatch in the spring after approximately 500 degree days. Alevins feed of their yolk sacks for about 300 degree days and use the gravel and rocks to hide from predators while they develop into fry and start to feed on insect larvae and smaller fish. Afterwards, juvenile fish can stay in their freshwater habitat for about 2 to 5 years and go through the development stages of fry and parr, up until they undergo smoltification and become smolts (McCormick et al. 1998; FAO 2020).

1.2 The current status of Atlantic salmon aquaculture production in Norway

Atlantic salmon is largely farmed both inland and offshore. Norway is the world's leading salmon-producing country and supplies almost 50% of the global production, hence playing a pivotal role in the Norwegian economy through employment, value creation and tax revenues (Lindland et al. 2019). In 2018, the total salmon aquaculture production in Norway reached a value of 1 354 941 tonnes (FAO 2018). Nonetheless, the long-term and sustainable development of the Norwegian salmon industry is imperilled by several challenges such as extended low-temperature periods making year-round intensive aquaculture production difficult, sea lice (*Lepeophtheirus salmonis*) infestation, escapees, growing concerns about wastewater management and environmental impacts, and animal welfare (Martins et al. 2010). Recently, efforts have been made towards developing solutions to address these challenges through development of novel production systems and protocols that can optimize the control of the rearing environment. Recirculating aquaculture systems (RAS) have been acknowledged as a promising solution to overcome these challenges (Dalsgaard et al. 2013; Ytrestøyl et al. 2020).

RAS are comprised of multiple components in one system such as rearing tanks, pipes, mechanical and biological filtration and offer numerous advantages compared with traditional smolt production in flow-through systems (Kristensen et al. 2009). It allows for flexible location of the production sites, water recirculation, more efficient waste management and nutrient recycling, improved biosecurity and disease control, prevention of escapees, and lower vulnerability to harsh and unpredictable environmental conditions (Dalsgaard et al. 2013; Summerfelt et al. 2016). Production of post-smolts up to 1 kg in RAS is being attempted as a way of producing more robust fish previous to sea-transfer (Hagspiel et al. 2018). Fish production in RAS is normally undertaken in high densities, with long water retention times and high feeding rates that lead to high organic loads and micro-particle build up, which in turn might favour the growth of opportunistic bacteria and the presence of pathogenic bacteria in the system (Rurangwa and Verdegem 2015; Liu et al. 2018). Therefore, efficient biosecurity

measures must be employed, where the use of routine disinfection is essential to ensure proper functioning of the recirculation system.

1.3 Peracetic acid

1.3.1 The use of peracetic acid in aquaculture production

Peracetic acid "PAA" (CH_3CO_3H) is the peroxide of acetic acid, and is a strong oxidative disinfectant commercially available as a quaternary equilibrium mixture of PAA, acetic acid, hydrogen peroxide (H_2O_2), and water. Its disinfectant activity is mainly achieved by the release of oxygen radicals that impair the transport and chemiosmotic function of the lipoprotein cytoplasmic membrane, resulting in oxidative damage of cell membranes (Liberti and Notarnicola 1999; Stampi et al. 2001; Kitis 2004; Marchand et al. 2012). For years, PAA has been widely used as a disinfectant in wastewater treatment, in food industries and hospital facilities (Kitis 2004; Marchand et al. 2012).

More recently, PAA has been regarded as a promising disinfectant for the enhancement of biosecurity in aquaculture due to its proven effectiveness against several microorganisms at low concentrations, short contact time, low dependency on pH, and rapid degradation into harmless residues as opposed to other chemotherapeutics formerly used in aquaculture such as malachite green and formaldehyde (Monarca et al. 2002; Meinelt et al. 2009; Sudová et al. 2010; Jussila et al. 2011). Furthermore, PAA does not seem to contribute to the formation of pathogen resistances. In general, its effectiveness against pathogens can be categorised as follows: bacteria > viruses > bacterial spores > protozoan cysts (Wessels and Ingmer 2013). PAA is also recognised as an alternative to H_2O_2 since it degrades faster and presents a lower effective dose against multiple pathogens (1-2 mg/L) than H_2O_2, making it safer for the biofilter and therefore more suitable for application in RAS (Pedersen et al. 2013; Liu et al. 2016; Pedersen and Lazado 2020).

Overall, these features underscore the advantages of using of PAA as a sustainable option for routine disinfection in aquaculture production. However, a comprehensive knowledge on the physiological impacts of PAA routine disinfection on the fish must be achieved to further support its use, as it currently remains fragmentary.

1.3.2 Peracetic acid toxicity

Products used for disinfection in aquaculture should present high efficacy towards the target pathogens while safeguarding fish health, food, worker and environmental safety. Although PAA-based disinfectants can be effectively used to reduce the prevalence of pathogenic diseases, the safe treatment margin of PAA is relatively narrow due to its unspecific mode of action (Pedersen et al. 2013; Wessels and Ingmer 2013). In order to prevent

deleterious effects to fish health, PAA toxicity has been studied in different fish species. The 24-h and 48-h median lethal concentration (LC50) values were reported to be 2.6 mg/L PAA and 1.6 mg/L PAA for yolk sac fry and swim-up fry channel catfish (*Ictarulus puntactus*), respectively, while the 24-h no observed effect concentration (NOEC) was 2.2 mg/L PAA to yolk sac fry and 1.3 mg/L PAA to swim-up fry (Straus et al. 2012). Another study documented the PAA 24-h LC50 and NOEC to fingerlings of different fish species (Straus et al. 2018). For instance, the LC50 was found to be 4.17 mg/L to rainbow trout (*Oncorhynchus mykiss*), 5.88 mg/L to largemouth bass (*Micropterus salmoides*) and 9.31 mg/L to blue tilapia (*Oreochromis aureus*), whereas the NOEC was 2.8 mg/L, 4.0 mg/L and 5.8 mg/L, respectively.

Moreover, PAA toxicity can be affected by the existing environmental conditions. Low-hardness waters can increase PAA acute toxicity. This association between water hardness and PAA toxicity was confirmed through observation of significantly lower 24-h LC50 values of PAA in zebrafish (*Danio rerio*) embryos in low-hardness water compared with high-hardness water (Marchand et al. 2013). Therefore, specific protocols of PAA application must be established according to the farmed species and rearing parameters (Straus et al. 2018).

1.3.3 Physiological consequences of the use of peracetic acid in fish

The use of PAA-based products in aquaculture substantially decreases the prevalence of pathogenic microorganisms. However, the potential short-term and long-term impacts of PAA exposure in fish physiology must be documented, as knowledge in this subject is rather limited (Liu, Straus, et al. 2017; Lazado, Haddeland et al. 2020). Two distinct strategies are typically adopted for PAA application in aquaculture: periodic or continuous PAA administrations, which result in pulse high concentration exposure or extended low concentration exposure, respectively. Therefore, different effects in fish physiology are likely to be seen from the two strategies (Liu, Straus, et al. 2017; Gesto et al. 2018).

So far, only a few studies have reported the physiological alterations in different fish species following PAA exposure. For instance, one study investigated the effects of periodic PAA administrations for 5 weeks in common carp (*Cyprinus carpio*), where following a significant initial rise, increases in the water cortisol concentrations became insignificant after repeated applications, suggesting that fish adapted to periodic PAA exposure (Liu, Pedersen, et al. 2017). In another study, the effects of periodic and continuous PAA applications on the performance of rainbow trout (*Oncorhynchus mykiss*) were compared (Liu, Straus, et al. 2017). Periodic PAA application at 1 mg/L led to increased water cortisol concentrations, which was not observed for the continuous PAA application at 0.2 mg/L. Nevertheless, progressively lower increases in water cortisol were observed for the periodic application protocol, suggesting habituation from the fish. Moreover, periodic applications inhibited biofilm formation and resulted in enhanced water quality, indicating that despite triggering a minor initial stress,

periodic applications are more advantageous than continuous applications. Later, Gesto et al. (2018) reported that rainbow trout were capable of assembling a physiological stress response to an acute stressor (chasing stressor) regardless of previous PAA exposure history - control, periodic exposure, and continuous exposure, showcased by a quick recovery in the levels of plasma cortisol, plasma lactate and brain serotonergic activity after stress was provoked. This observation allowed to conclude that the former observations of lower cortisol levels after repeated PAA exposure (Liu, Pedersen, et al. 2017; Liu, Straus, et al. 2017) were indeed a classic sign of habituation.

The effects of PAA exposure in Atlantic salmon smolts were very recently reported, with some of the investigated systemic and mucosal stress indicators being distinctly affected (Soleng et al. 2019). Plasma cortisol dynamics were in line with previous reports, whereas plasma glucose and lactate levels were not significantly affected by previous PAA exposure when fish were re-exposed to PAA. The antioxidant defence system proved to be more responsive in the gills than in the skin, based on transcriptional changes. In addition, total antioxidant capacity in plasma was increased, in particular with higher concentrations of PAA, probably to counteract the internal redox imbalance elicited by PAA, an abundant source of exogenous reactive oxygen species (ROS). Lazado, Haddeland et al. (2020) investigated the effects of PAA treatment at the concentrations of 0.6 mg/L and 2.4 mg/L in the skin of Atlantic salmon. While no lesions or wounds were observed in any of the groups, fish treated with PAA exhibited rougher epidermal surface compared with the control group, particularly following a second treatment. In addition, Lazado, Sveen, et al. (2020) studied the transcriptional, morphologic and physiological parameters in salmon smolts exposed to PAA and crowding stress, and reported that PAA exposure did not substantially affect the external welfare, number of mucous cells in the skin and gills, as well as plasma metabolomics. Conversely, the transcriptional profile in the gills proved to be more sensitive to PAA than in the skin, showing a strong transcriptional response in the gills at 4h post-exposure and a striking recovery after 2 weeks. Moreover, crowding altered the transcriptional response to PAA, resulting in a pattern of downregulation in the gills and upregulation in the skin. A different experiment also compared the effects of periodic and continuous exposure to PAA in rainbow trout (Liu et al. 2020). The endogenous total free radicals (TFR) were significantly increased, and subsequently TAC activity was increased in the gills and serum. Minimal gill hyperplasia was found in periodically exposed fish only, while the continuous exposure protocol led to reduced ceruloplasmin and anti-protease activity in serum. Although both protocols elicited antioxidant defence mechanisms in the fish, the authors described the periodic exposure protocol as preferable, as the time between applications seemed to allow fish to assemble effective antioxidant defences.

These studies have provided valuable insights into the potential impacts of PAA and highlighted the capacity of fish to adapt to the physiological demands of PAA disinfection.

1.3.4 Application and degradation of peracetic acid in RAS

The efficacy of PAA disinfection under practical rearing conditions largely depends upon the existing environmental conditions. Water parameters in aquaculture are affected by various factors including water source and composition, species reared, feeding rate, stocking densities, water treatment units, and degree of water reutilization (Pedersen et al. 2009; Liu et al. 2014). The residual levels of PAA in the water are a defining factor of the disinfection efficacy, since the active concentration of PAA is determined not only by the applied dose, but also by the product's stability and exposure time (Pedersen et al. 2013). Although the decay of PAA in aqueous solutions normally follows a first order reaction kinetics, different water parameters are seen in distinct aquaculture systems, and therefore PAA degradation is expected to differ between them (Liu et al. 2016). Organic matter content greatly affects PAA's rate of decay, with increasing levels of organic matter resulting in higher disinfection demand of PAA. Moreover, parameters such as salinity, water temperature, existing biofilm, light and stocking density affect the decay of PAA in different degrees, whereas higher dosages of PAA only slightly reduce the rate of decay (Pedersen et al. 2013; Pedersen and Lazado 2020). As for pH, when within a range of 5.5 – 8.2, pH seems to have negligible influence in the degradation of PAA (Yuan et al. 1997). PAA half-life becomes significantly shorter in brackish water and saltwater RAS when compared with freshwater facilities, making it necessary to use higher PAA dosages or multiple applications to achieve effective concentrations when performing treatments in brackish water or saltwater facilities (Liu et al. 2014; Pedersen and Lazado 2020).

One additional aspect to consider in the disinfection of RAS with PAA, is that due to the elevated water retention time, typical of this type of production system, PAA can accumulate in the system when high concentrations are used and react with the nitrifying bacteria present in the biofilter, potentially harming the bacteria and impairing the nitrification process, which in turn results in the accumulation of ammonia and nitrite in the system. Nevertheless, in treatments where concentrations of 1 mg/L (selected concentration in the present study) or less are used, PAA rapidly degrades and thus should not affect the biofilter performance (Pedersen et al. 2009).

1.4 Fish welfare in aquaculture production

As aquaculture currently provides more than 50% of the fish consumed worldwide and the industry continues to grow, considerable attention has been given to the welfare of farmed fish (Huntingford and Kadri 2014). Due to the fact that fish are vertebrates, sharing a set of

features with the more frequently produced species of mammals and birds, several welfare principles and indicators applied to fish were adopted from the study of these animal groups (Huntingford and Kadri 2014). When discussing animal welfare, three comprehensive and overlying ethical concepts are typically considered (Fraser et al. 1997):

1) Nature-based definitions: animals should be able to live a natural life throughout their development by expressing their natural behaviour, and being able to use their natural adaptations and competences.
2) Feeling-based definitions: animals should have access to positive experiences. Negative experiences such as prolonged and intense fear, pain, and other negative experiences should be absent.
3) Function-based definitions: animals should function well, in terms of showing good health and growth, as well as proper function of physiological and behavioural systems.

However, as fish have a long, divergent evolutionary history to other animal groups and have adapted to very distinct and challenging conditions that comprise the aquatic environment, they are not directly comparable to mammals and birds (Huntingford and Kadri 2014). Thus, specific guidelines on how to define, evaluate and guarantee fish welfare are needed. Some authors question the validity of the feeling-based concept to assess the welfare of fish, as they argue that fish do not possess the capacity of suffering (Rose 2002; Rose 2007; Rose et al. 2014). This argument is mainly based on the fact that fish have a small and rudimentary brain compared to their mammalian counterparts, lacking structures that establish numerous higher mental processes in humans. Other authors have contended this view, defending that fish behaviour is much more complex than generally thought and that a substantial degree of homology and functional similarity between fish and mammals exists and should be considered (Braithwaite and Huntingford 2004; Chandroo et al. 2004). In fact, the capability of some species of fish to undergo experiences associated with negative affective states like suffering is becoming increasingly evident (Braithwaite and Ebbesson 2014).

On the other hand, the welfare status of fish can be assessed in an easier and more precise manner following the nature and function-based definitions. In order to accurately assess welfare, a multiple parameter approach is typically employed by researchers, relying on variables that account for health, physical, physiological and behavioural status (Huntingford and Kadri 2014). Detection of injuries and diseases, as well as changes in ventilation rate, food intake, growth, colour and swimming activity are some of the classical indicators used to monitor fish welfare. Recently, different molecular tools such as genomics are being widely used to evaluate the welfare of fish (Prunet et al. 2012).

In modern intensive aquaculture, it is somewhat inevitable that situations causing fish to experience stress occur (Conte 2004). Besides, the stress response is intrinsically related to

the behavioural, physiological and health aspects of fish and therefore stress responses are an important indicator for welfare assessment (Iwama 1998; Huntingford and Kadri 2014).

1.5 The concept of stress in fish

Several approaches are used to define stress. Selye (1973) defined stress as “the nonspecific response of the body to any demand made upon it “. It is also defined as the reaction of the cell or organism, to a given challenge placed upon it, in a way that results in the extension of a physiological state past the typical resting state (Iwama 1998). Moreover, stress can be regarded as a state of compromised homeostasis that is restored by a collection of adaptive mechanisms (Barton 2002).

The stress response was initially described by Selye (1950) as “General Adaption Syndrome” (GAS), which consists of three stages: first the alarm reaction occurs, which is the earliest response to the stressful stimulus, followed by a resistance phase where the organism tries to adapt to the disturbance in order to re-establish homeostasis. In the event of the organism being unable to deal with the existing stressors, the third stage takes place, where exhaustion occurs, possibly leading to disease or death. Regardless of some limitations inherent to this theory, it remains pertinent for the discussion of the stress response in fish which is still considered as a three stage process (Schreck and Tort 2016).

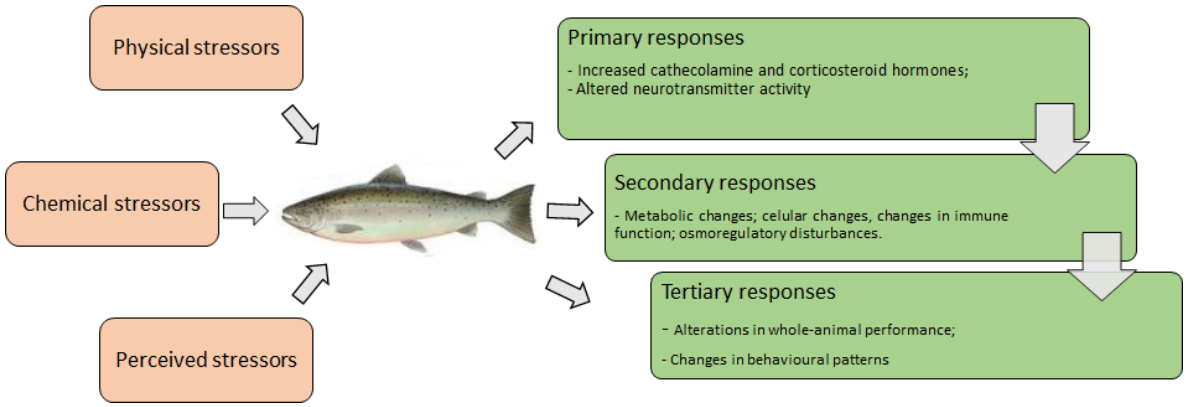

Figure 4 - Representation of some physiological features of the stress response in fish. Adapted from Barton (2002); salmon: Mirimachi Salmon Association, https://miramichisalmon.ca/education/atlantic-salmon/

The initial response is characterised by the perception of an altered state, leading to a neuroendocrine response that is part of the general stress response in fish (Iwama 1998). This response includes the release of stress hormones into the circulation, such as catecholamines and cortisol. Catecholamines are released from the chromaffin tissue, located in the anterior kidney, and from the endings of adrenergic nerves. Cortisol is released from the anterior kidney, following stimulation from the hypothalamic-pituitary-interrenal (HPI) axis, mostly in

response to increasing levels of adrenocorticotrophic-hormone (ACTH), even though cortisol shows responsiveness to several others pituitary hormones (Wendelaar Bonga 1997; Barton 2002). The secondary response includes the various biochemical and physiological changes related to stress and is chiefly influenced by the stress hormones released during the primary stress response (Iwama 1998). Examples of secondary responses are changes in plasma and tissue ion or metabolite levels, heat-shock proteins, and haematological features, all of them associated with physiological adjustments like respiration, metabolism, hydromineral balance, acid-base status, immune function and cellular responses (Barton 2002). These physiological changes facilitate behavioural adaptations such as escape, freezing or alertness, which increase the chances of survival. The tertiary stress response stage is characterised by changes at the whole-organism and population levels in response to stress. In order to cope with the higher energy demands associated with continued stress, the animal may need to redirect energy substrates from vital processes, namely reproduction and anabolic processes such as growth. These adaptations will invariably impact animal performance (Wendelaar Bonga 1997; Barton 2002).

Although the stress response is an integral part of life and its benefits in coping with short-term stressors are undeniable, such physiological mechanisms may be impaired and eventually become harmful to the fish (Pottinger 2010). Mild forms of stress that may not look detrimental at first, may well affect the performance and welfare of farmed fish over long periods of time (Barton and Iwama 1991; Schreck and Tort 2016) and thus, stress responses must be assessed as indicators of the welfare status of farmed fish.

1.6 Indicators used for the assessment of fish welfare

1.6.1 Cortisol

Cortisol is the main circulating steroid hormone in fish, and the rise of plasmatic levels represents the most-studied element of the stress response in fish (Wendelaar Bonga 1997; Mommsen et al. 1999). Cortisol is synthesised in the interrenal cells, which are disposed in layers, strands and cords around the walls of the posterior cardinal veins and its branches running through the head kidneys (Wendelaar Bonga 1997). The secretion of cortisol is under regulation of the HPI axis, with ACTH being the major secretagogue for cortisol. However, alpha-Melanocyte-stimulating hormone (α-MSH) seems to have some corticotropic activity when potentiated by β-endorphin (Balm et al. 1995).

Cortisol has a crucial role in the maintenance of homeostasis under stress conditions, with the gills, liver and intestine as target organs, combining both mineralocorticoid and glucocorticoid functions. In regard to its mineralocorticoid function, cortisol regulates hydromineral balance by promoting the differentiation of chloride cells, responsible for ion-transport in the gills, and by stimulating the specific activity of ion-transporting enzymes,

primarily sodium–potassium adenosine triphosphatase (Na-K+-ATPase), in gills, kidneys and intestine (Wendelaar Bonga 1997), whereas to its glucocorticoid function it has effects in whole body metabolism, promoting gluconeogenesis in the liver and lipolysis in the muscles and liver, which may be of critical importance to cope with increased energy demands (Barton and Iwama 1991; Mommsen et al. 1999). Nonetheless, prolonged secretion and elevated levels of cortisol under long-lasting stress events are detrimental to fish health and overall performance, impairing immune system function and anabolic processes such as growth and reproduction by diverting energy resources from these kinds of processes to meet more immediate energy requirements (Pickering and Pottinger 1989; Wendelaar Bonga 1997).

Usually, the degree and duration of plasma cortisol levels increase reflects the magnitude and duration of a stressor (Barton and Iwama 1991). In addition, the lag time of its release which allows precise measurement of basal levels and stressor-induced increases, and its functional role in physiological processes makes cortisol a first choice indicator for researchers in the study of the effects of acute stress in fish (Barton 2002). In resting or undisturbed fish, the basal circulating cortisol levels are typically lower than 30-40 ng/mL and should ideally be <5 ng/mL. Post-stress elevations of plasma cortisol in salmonids normally range between 40-200 ng/mL (Barton and Iwama 1991). After exposure to an acute stressor, cortisol concentrations classically rise in a matter of minutes, while the return to basal levels can take up to one or more hours. This response dynamic has been described for several fish species confronted with multiple stressors like handling, exposure to heavy metals and organic pollutants, drastic water temperature changes and predators (Wendelaar Bonga 1997). Conversely, when a stressor is chronic, high levels of cortisol are sustained but below peak levels. Resting levels of cortisol can however suffer variation to some extent during distinct life stages such as smoltification, final gonadal maturation and spawning (Wendelaar Bonga 1997; Pottinger 2010). Environmental factors such as water temperature and salinity, and endogenous factors such as nutritional state and diurnal or seasonal cycles alter the clearance of cortisol, and will consequently have an impact on the cortisol levels. Therefore, different external and internal factors must be taken into account when interpreting the physiological stress responses of fish (Barton and Iwama 1991; Barton 2002).

Studies have shown that PAA exposure triggers an increase in cortisol levels followed by a form of habituation in response to repeated PAA applications (Liu, Straus, Pedersen, et al. 2017; Liu, Pedersen, Straus, et al. 2017; Gesto et al. 2018). Moreover, in the first-ever study performed with Atlantic salmon exposed to PAA, Soleng et al. (2019) reported that previous history of PAA exposure did not disrupt stress-induced cortisol dynamics.

In conclusion, cortisol is the most widely accepted and used indicator of stress in fish. Due to its responsiveness to acute stressors, its physiological significance in fish health and

ease of sampling, cortisol is ideal for monitoring stress responses in fish (Barton and Iwama 1991; Wendelaar Bonga 1997; Mommsen et al. 1999)

1.6.2 Glucose

Glucose is a metabolite that plays an essential role on the secondary stress response in fish. The circulating glucose levels are a function of glucose production and clearance (Iwama 1998). Multiple stressors are known to trigger elevations in glucose concentrations, thus glucose is frequently used as an indicator of the metabolic effects of stress in fish (Barton and Iwama 1991). The rise of plasma glucose levels during stress is a vital metabolic process that enables fish to meet the increased energy requirements. In fact, although glucose does not appear to be as critical to fish general metabolism and its endocrine regulation is not as well understood as in mammals, glucose is particularly important for fish metabolism in the presence of stressful stimuli, supplying energy substrates to tissues like the brain, gills and muscles (Mommsen et al. 1999; Polakof et al. 2012).

The liver is the main source of glucose production, which is obtained either by glycogenolysis (break down of glycogen into glucose) or gluconeogenesis (synthesis from lactate, glycerol and other amino acids) (Wendelaar Bonga 1997; Polakof et al. 2012). Although it is yet to be entirely understood, a cause-effect association seems to occur between the primary stress responses and the production of glucose (Barton and Iwama 1991; Iwama 1998). Cathecolamines seem to prompt the initial release of glucose, mainly through stimulation of glycogenolysis, whereas cortisol later contributes to maintain high levels of glucose, either by promoting gluconeogenesis or regulating peripheral glucose uptake (Leach and Taylor 1980; Wendelaar Bonga 1997).

As with cortisol, multiple stressors like crowding and exposure to heavy metals lead to increased plasma glucose levels (Basrur et al. 2010; Grassie et al. 2013). However, the kinetics of glucose typically display a delayed and more persistent pattern compared with cortisol. For instance, brown trout (*Salmo trutta*) exposed to 2 minutes of handling presented a peak concentration of glucose at 4 hours and stabilized after 72 hours (Pickering et al. 1982), while plasma glucose levels in Atlantic salmon subjected to a 2 hour transport did not recover to resting levels following a 48 hours recovery period (Sandodden et al. 2001).

Concerning PAA, Soleng et al. (2019) concluded that prior history of treatment with PAA in the concentrations of 0.6 mg/L and 2.4 mg/L alone did not significantly affect glucose levels and consequently glucose metabolism in salmon.

1.6.3 Lactate

Lactate is an energy metabolite involved in the secondary stress response and is often used as an indicator of stress in teleost fish (Barton and Iwama 1991). Lactate is formed by the dissociation of lactic acid, which in turn accumulates via glycogenolysis that provides the

majority of ATP necessary during anaerobic activity (Kieffer 2000). Elevated plasma lactate concentrations are reached in response to numerous acute stressors such as handling, transport, hypoxia, forced exercise, osmotic and temperature shocks, social stressors or exposure to water pollutants (Wendelaar Bonga 1997), with such rises resulting largely from anaerobic activity of white muscle during periods of strenuous exercise (Pankhurst and Dedual 1994). Thus, it can be concluded that lactate concentrations are particularly responsive to stressful events that promote a rise of activity levels or a reduction of oxygen availability.

Lactate seems to be a preferred substrate for gluconeogenesis in fish, underscoring its metabolic importance during stressed states. Moreover, lactate gluconeogenesis is induced by glucocorticoids, suggesting an intricate relationship between the hormonal components of the stress response. However, it is arguable whether lactate is used for glucose production or maintenance of liver glycogen in fish after stress (Mommsen et al. 1999).

Soleng et al. (2019) reported that the tested concentrations of PAA, duration of exposure and re-exposure did not significantly change the plasmatic lactate levels in salmon.

1.6.4 Total antioxidant capacity

PAA-based disinfectants contain PAA and H_2O_2, two chemicals with oxidative properties, meaning PAA products are a natural source of ROS (Liu et al. 2020). Although ROS are produced as natural by-products of several metabolic pathways (Sopinka et al. 2016; Birnie-Gauvin et al. 2017), when the production of ROS surpasses the balancing capacities of antioxidant defences, oxidative stress occurs and damage to biological molecules such as lipids, proteins and DNA may arise (Lesser 2006). Antioxidants can be mobilised to prevent the negative impacts of ROS, either by inhibiting the formation or by scavenging ROS (Sopinka et al. 2016). In fact, studies have documented the increase in TAC levels as an indicator of the mobilisation of antioxidants to counterweight redox imbalance following oxidative stress (Wu et al. 2017; Soleng et al. 2019) . Nevertheless, the antioxidant defences in fish respond differently to specific types of xenobiotics and diverge substantially between species and organs within a species (Martínez-Álvarez et al. 2005).

As PAA-based products are a known source of exogenous ROS, it is expected that exposure to PAA might prompt a transitory state of oxidative stress in fish before full degradation takes place. Increases in serum TAC levels by 8-fold and 5-fold were documented in rainbow trout subjected to periodic and continuous PAA exposure compared with unexposed trout (Liu et al. 2020). Furthermore, elevations in TAC levels were reported in Atlantic salmon following re-exposure to PAA (Soleng et al. 2019). These observations suggest that antioxidant defences were triggered to protect the fish against the oxidative stress caused by PAA exposure and maintain homeostasis.

1.6.5 Mucosal Immunity

Despite the evolutionary divergence of approximately 450 million years, the mucosal organs and tissues of teleost fish are rather similar to their mammalian counterparts in terms of structure and functions (Beck and Peatman 2015). As fish are in permanent and intimate contact with the aquatic environment, they are continuously presented with biological, chemical, and physical challenges that pose constant pressure to the adaptive and protective mechanisms of fish (Cabillon and Lazado 2019). Thus, mucosal surfaces in fish constitute a crucial first line of defence against the constant changes in the external environment, acting as a physical barrier and playing a vital role in teleost immunity (Salinas 2015).

The gills, skin and gut represent the most comprehensively studied mucosal organs in fish, where mucosal-associated lymphoid tissues (MALT) are found and play a crucial role in immunity as secondary lymphoid organs (Cabillon and Lazado 2019). Moreover, a nasal-associated lymphoid tissue (NALT) has been recently discovered in the olfactory organ of teleost fish, suggesting an involvement in important defence mechanisms besides its extensively described sensory functions (Tacchi et al. 2014). Even though these mucosal tissues exhibit distinct physiological functions, they share microanatomical features such as an organised epithelial surface with lamina propria or supporting stromal tissues, a vascular supply system, and resident immune cells. Furthermore, they are covered by a mucous layer, which is secreted by mucous/goblet cells and constitutes a physical, chemical, and biological barrier with potent inhibitory activity towards numerous pathogens, and harbour resident microbiota that provide additional defence via production of H_2O_2, bacteriocins and antimicrobial peptides (Salinas 2015; Cabillon and Lazado 2019).

The health status of the mucosae often has implications in the overall health status of the fish, evidencing the significance of mucosal health in aquaculture production. In addition, the integrity and function of mucosal surfaces is reliant on the existing environmental conditions and shows remarkable responsiveness to modifications in parameters such as water temperature, photoperiod, pH, oxygen saturation, and turbidity in the form of molecular and phenotypic changes (Beck and Peatman 2015). This makes mucosal surfaces sensitive biomarkers for the evaluation of the prevailing rearing conditions (Lazado 2020).

1.6.6 Histological biomarkers for welfare assessment

The correct way to accurately estimate the welfare status of fish is a never-ending debate, with agreement on definitions or assessment methodologies still far from being accomplished (Huntingford and Kadri 2014). Biomarkers are measures of suborganismal reactions in organisms or exposed biological systems that can exhibit exposure to, or the impacts of chemicals present in the environment (Au 2004).

Histopathology embodies a group of biomarkers that allow the examination of specific target organs, including gills, kidney and liver, which develop vital functions such as respiration, excretion, accumulation and biotransformation of xenobiotics (Gernhöfer et al. 2001). Histopathological biomarkers integrate biotic factors and water quality in a holistic interpretation of fish health, making them reliable indicators of environmental stress (Poleksic et al. 2010). One advantage in the study of histological biomarkers is its intermediate position in terms of the degree of biological organization. Histological alterations develop as an intermediate response to sub-lethal stressors, and thus histology can be used for the rapid detection of the effects of chemical irritants in different tissues and organs, which have implications to the health and fitness of individual fish, and allow to infer the possible effects to the population (Bernet et al. 1999; Au 2004; Camargo and Martinez 2007).

Although invasive and time consuming, histopathology can be defined as a gold standard methodology to investigate the toxicological effects of different chemical irritants. Nonetheless, to correctly assess the health and welfare of fish using histopathology techniques, it is of utmost importance to comprehend the pathological significance of the observed alterations, namely their implications on organ function and fish survival (Bernet et al. 1999).

1.6.6.1 Histopathology and tissue morphometric analysis of the gills

The gills are a multipurpose organ that acts as the main site for respiration, acid/base balance, metabolism of circulating hormones and nitrogen excretion. Therefore, the branchial epithelium is crucial in a variety of physiological responses to internal and environmental changes. The gill apparatus is composed of four branchial arches on each side, supporting two rows of primary filaments, the functional units of the gill. Primary filaments are covered by secondary lamellae, where gaseous exchange takes place, mainly through counter current exchange of blood flowing in the opposite direction from the external water (Evans et al. 2005; Olson 2011).

As the gill surface represents more than half of the entire body surface and is permanently in intimate contact with the external environment, it becomes particularly susceptible to multiple irritant materials present in the water (Roberts 2012; Wolf et al. 2015). Structural changes on the gill are caused by numerous irritants, including heavy metals, organic pesticides, low pH, gas supersaturation, suspended solids, microbiological infections and parasites (Mallat 1985; Strzyzewska et al. 2016). Hence, histopathology is considered a sensitive and accurate indicator of gill health (Au 2004; Wolf et al. 2015; Strzyzewska et al. 2016). Since the gills have a reasonably small number of component cells and tissues, the extent of pathological responses are limited. The earliest and most commonly observed changes in response to low levels of external irritants typically are hypertrophy of individual

lamellar cells and increased volume of mucus secretion over the epithelial surface, and are indicative of changes in membrane permeability at the cellular and tissue level. However, depending on the type of irritant stimulus, in case its magnitude increases, four distinct alterations can appear, namely lamellar oedema, epithelial necrosis, lamellar hyperplasia and lamellar fusion, although a combination of all four is frequently observed in more advanced stages of lesion (Roberts 2012; Strzyzewska et al. 2016).

Distinct protocols of PAA exposure have been reported to cause histopathological changes in different aquatic animals. Straus et al. (2012) described severe degeneration of the gill epithelium in swim-up fry channel catfish exposed to 2.2 mg/L of PAA, culminating in large-scale acute mortality of the fish. Another study reported dose dependent changes in the gills of grass carp exposed to PAA such as lamellar fusion, lamellar clubbing, and epithelial lifting (Chupani et al. 2014). Notwithstanding, such alterations were suggested to be reversible, since the main structure of the gill appeared unaffected. Moreover, signal crayfish (*Pacifastacus leniusculus*) exposed to 10 mg/L of PAA for seven days presented hemocytic infiltrations, abnormal lamella tips, and disorganization of epithelial cells in the gills (Chupani et al. 2016). In contrast, another study showed that mirror carp treated twice per week with 1 mg/L PAA exhibited healthier gill tissue than fish from the control group (Liu et al. 2018). More recently, Liu et al. (2020) described that biweekly exposure to 1 mg/L PAA resulted in minimal gill hyperplasia.

Morphometric assessment, typically involving the measurement or quantification of particular tissues, cell populations or subcellular constituents, has been extensively performed for the study of gill-tissue alterations (Hughes and Perry 1979). Such assessments are usually executed using light microscopy by counting points of interest, for example for counting the number of chloride cells (Maina 1991). Other morphometric evaluations such as estimating the respiratory surface area in response to distinct values of partial pressure of oxygen (PO_2) (Saroglia et al. 2002), or assessing the effects of short-term copper exposure on the gill epithelial thickness (van Heerden et al. 2004) have also been frequently performed and provide relevant information on the gill's function in gas exchange and subsequently on gill health.

Histopathology and morphometric analysis can thus be employed to investigate the effects of PAA exposure in overall gill health and function.

1.6.6.2 Tissue morphometric analysis of the skin

The teleost skin constitutes the first line of defence against the external environment, allowing normal internal physiological regulation. It contacts directly with toxic chemicals, parasites, and pathogens in the water, thus its condition is a defining factor in many pathological processes (Roberts 2012). In normal environmental conditions, the fish integument is well adapted as a limiting barrier. However, the skin is a delicate organ and thus

susceptible to damage in culture tanks or contaminated waters and to the subsequent osmoregulatory disturbance. Alterations of the mucous coat or underlying layers could impair the normal physiological function of the internal environment of the fish. Such changes result in abnormal behaviour, disease, or death. In fact, lesions in the skin are typically the first observed clinical signs of disease in fish (Roberts 2012; Salamat and Zarie 2016).

The examination of fish skin as a biomarker in water quality tests or environmental risk assessment programmes is not always employed, as it can be hard to assess the acute effects of toxic chemicals in the skin (Salamat and Zarie 2016). In addition, the uptake of toxicants by the skin is usually less significant than uptake by the gills, since the skin classically represents a smaller surface area, a thicker and more impermeable diffusion barrier, slower transport of water, and lower blood flow (Capkin et al. 2009).

In the past, some histomorphological features of the skin have been measured to evaluate the impacts of xenobiotic exposure in the health of fish (Sanchez et al. 1998; Buchmann et al. 2004; Vatsos et al. 2010). Furthermore, morphometric analysis has been recently used to study the impacts of PAA exposure. A significant increase in the number of mucous cells was reported in the skin of Doctor fish (*Gera rufa*) exposed to PAA (Sirri et al. 2013). Conversely, Liu et al. (2020) described significantly reduced mucous cell density in the epidermis of trout in response to continuous exposure to 0.2 mg/L PAA. In a study regarding the effects of PAA in the skin of salmon, no significant differences were found in mucous cell area and mucous cell density between treatment groups (Lazado, Haddeland, et al. 2020).

1.6.6.3 Tissue morphometric analysis of the olfactory rosette

The olfactory system is a critical chemosensory system in fish, implicated in multiple functions such as foraging food, finding sexual and social partners, or identifying and evading predators or other threats. As a result, the fish olfactory system is responsive to numerous molecules, ubiquitous in aquaculture, like amino acids, peptides, nucleic acids, bile acids and steroidal compounds (Hansen and Zielinski 2005). In teleost fish, the olfactory organ is a paired structure positioned in the snout, with each olfactory organ composed by an olfactory cavity connected to the exterior via the anterior and posterior nostrils, which allow water to flow into the nasal cavity as the fish swims (Laberge and Hara 2001).

The olfactory epithelium, also known as olfactory rosette due to its rose-like structure, consists of a set of olfactory lamellae that raises from the floor of the olfactory cavity. The rosette structure and number of olfactory lamellae are species-specific, and in salmonids the olfactory epithelium displays a multi-lamellar rosette covered by sensory and non-sensory epithelium (Hansen and Zielinski 2005). Three distinct types of olfactory sensory neurons (OSNs) - ciliated, microvillous and crypt cells, are found within the sensory epithelium. Basal cells are also present and support the olfactory epithelium by acting as a reservoir for

development and regeneration. Supporting cells are typically dispersed among the OSNs, whereas goblet cells are abundant in the non-sensory epithelium of the nasal cavity but can be occasionally found within the sensory epithelium (Hansen and Zielinski 2005; Hamdani and Døving 2007).

As the olfactory sensory neurons are in direct contact with the external environment, the olfactory epithelium of fish is highly sensitive to water contaminants (Kasumyan 2004; Ghosh and Chakrabarti 2010). Several authors have successfully used morphometric measurements to investigate the structural impacts of xenobiotics in the olfactory organ of fish (Sandahl et al. 2006; Tierney et al. 2010; Lazzari et al. 2017). To the best of the author's knowledge, there is no available data on the consequences of PAA exposure to the olfactory rosette. Notwithstanding, since the olfactory epithelium is highly responsive to different xenobiotics, morphometric analysis of the olfactory rosette should provide valuable insights on the impacts of PAA exposure in salmon.

1.6.7 Gene expression

In eukaryotic organisms, the entire genetic information is contained in the nucleus of every single cell. Only a fraction of the genetic information is expressed depending on the function of the cell, its physiological status and several environmental factors (Orphanides and Reinberg 2002). Gene expression encompasses transcription, translation and the turnover of mRNA (messenger RNA) and proteins (Buccitelli and Selbach 2020). Therefore, gene expression within the cell is regulated at numerous different levels through various mechanisms, which control and secure the activation and expression of specific genes (Orphanides and Reinberg 2002). Transcription, the process where information is transferred from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA), takes place in the nucleus of eukaryotic cells, and represents the most important level for the regulation of gene expression (Orphanides and Reinberg 2002; Buccitelli and Selbach 2020). Since stressors and even pathological states can induce an alteration in the expression of a specific group of genes, the same genes can be used to assess the welfare status of a given organism (Prunet et al. 2012).

1.6.7.1 Stress related gene expression

When facing a stressor, cells have the capacity of activating protective and reparative mechanisms through regulation of gene expression. Oxidative stress indicators are becoming more and more employed as biomarkers to investigate the mechanisms of various stressors and their physiological impacts in fish (Birnie-Gauvin et al. 2017). As exposure to PAA-based disinfectants likely induces oxidative stress via accumulation of ROS, the measurement of transcriptional levels of both enzymatic antioxidants and heat-shock proteins (hsps) can be

used as indicators to assess the degree of oxidative stress caused by PAA, a compound with oxidative properties (Sopinka et al. 2016).

In order to avoid or minimize the deleterious effects of exceeding levels of ROS, organisms possess protective mechanisms against oxidative stress, namely enzymatic and non-enzymatic antioxidants (Doyotte et al. 1997; Sies 1997; Lesser 2006). Antioxidants such as *glutathione peroxidase* (*gpx*), *glutathione reductase* (*gr*), *glutathione S-transferase* (*gsta*), *manganese superoxide dismutase* (*mnsod*), *copper/zinc superoxide dismutase* (*cu/znsod*) and *catalase* (*cat*) are integrant part of the fish antioxidant defence, and thus are frequently used as oxidative stress biomarkers in fish (Tkachenko et al. 2014; Birnie-Gauvin et al. 2017; Lazado and Voldvik 2020). *Superoxide dismutases*, *catalases*, and *glutathione peroxidases* are the three main classes of antioxidant enzymes and constitute the first line of defence against ROS by using them as substrates (Sies 1997; Lushchak 2016). *Superoxide dismutases* catalyse the dismutation of superoxide radicals to H_2O_2 and O_2 to neutralise oxygen radical-mediated toxicity following a stressful stimuli (Chalmers et al. 2018). *Cat* will then scavenge H_2O_2 and catalyse its conversion to H_2O and O_2, while *gpx* eliminates H_2O_2 in the presence of reduced glutathione (Doyotte et al. 1997; Lesser 2006). *Gr* and *gsta* are in turn part of the second line of defence against ROS, and assist the enzymes of the first line of defence. *Gr* regenerates the reduced glutathione, while *gsta* is responsible for the transport and elimination of reactive compounds (Sies 1997).

The expression of antioxidant enzymes in fish was shown to be significantly modulated by a variety of environmental stressors (Doyotte et al. 1997; Jiang et al. 2014; Vera and Migaud 2016; Chalmers et al. 2018). So far, very limited information on how PAA exposure modulates the expression of antioxidant enzymes in fish is available. Soleng et al. (2019) reported a tissue-specific pattern of gene expression in response to PAA exposure, with the gills appearing to be more responsive than the skin. While initial exposure did not alter the transcript levels of any of the antioxidant genes, a second exposure induced increased expression of *gpx* in both tissues, as well as increased expression of *mnsod* and *gr* in the gills, indicating that PAA exposure triggered mild oxidative stress.

Hsps are highly conserved cellular proteins that have been described in every organism including fish (Iwama 1998; Basu et al. 2002). In unstressed cells, these proteins are expressed in a constitutive manner and are actively involved in protein metabolism to maintain cellular homeostasis (Iwama et al. 2004; Deane and Woo 2011). Hsps are categorised into different families based on the approximate molecular mass (kDa) of the protein. From all the families of Hsps, three have been comprehensively studied - *hsp90* (85–90 kDa), *hsp70* (68–73 kDa) and low-molecular-mass *hsps* (16–24 kDa). *Hsp70* actively supports the folding of nascent polypeptide chains, acts as a molecular chaperone and facilitates the repair and degradation of altered or denatured proteins, whereas *hsp90* has an essential role in

supporting various components of the cytoskeleton, enzymes and steroid hormone receptors. As for low-molecular-mass *hsps*, these undertake species-specific functions (Basu et al. 2002; Iwama et al. 2004).

In recent years, it has become increasingly evident that the expression of hsps is upregulated not only by exposure to thermal stress, but also in response to a variety of biotic, abiotic and chemical stressors, as a mechanism to provide the cells with additional protection and maintain homeostasis (Ackerman et al. 2000; van der Oost et al. 2003; Deane and Woo 2011). Hsps are useful biomarkers of stress because they are rapidly induced by multiple stressors (Feder and Hofmann 1999). However, the extent of the heat shock response is reliant on the degree and duration of the stressor, and history of acclimation to previous stressors (Iwama et al. 2004). Within the hsp family, *hsp70* and *hsp90* have been employed in numerous studies as biomarkers to assess the occurrence of cellular stress in fish exposed to different unfavourable environmental conditions. Increased hsp expression has been described in fish exposed to industrial effluents, polycyclic aromatic hydrocarbons (Vijayan et al. 1998), heavy metals (Rajeshkumar et al. 2013) and pesticides (Hassanein et al. 1999). In regard to aquaculture production, Giri et al. (2016) found increased *hsp90* levels in the liver of rohu (*Labeo rohita*) after 28 days of exposure to sub-lethal concentrations of cadmium. High stocking densities were shown by Gornati et al. (2004) to alter the expression of hsps in European seabass (*Dicentrarchus labrax*, L.). Moreover, common carp exposed to Di-n-butyl phthalate showed altered expression of *hsp70* in the gills and liver (Agus et al. 2015), and Atlantic salmon revealed increased *hsp70* expression following treatment with H_2O_2 (Vera and Migaud 2016).

Alterations on the expression pattern of stress related genes can provide early and sensitive information on the magnitude of stress that certain stressors induce in fish.

1.6.7.2 Immune related gene expression

Since several resources need to be redistributed in order to cope with a stressful event, such metabolic adjustments can impair other biological processes, namely the immune system (Tort 2011). Some mechanisms of the defence repertoire may be hindered or diminished by stress, resulting in a transitory state of immune suppression, an extensively studied tertiary stress response. However, stress may also have stimulatory effects on the immune system of fish, which can be seen as secondary and adaptive responses, but are more difficult to detect and therefore less documented (Barton and Iwama 1991; Wendelaar Bonga 1997). While circumstances where the presence of a chronic stressor of low intensity but prolonged in time are unusual in nature, those same circumstances can be more commonly found in aquaculture. In situations where a chronic stressor persists, energy and resources need to be allocated to the stress response. As a result, resources will become insufficient for various processes of

the immune system that are energetically costly such as the production and differentiation of leukocytes, the synthesis of complement proteins, and the production of antibodies, thus becoming less efficient and culminating in immune suppression (Tort 2011).

Interleukins represent a subset of cytokines that are implicated in the intercellular coordination of the immune response. Several of the interleukins known in fish are direct homologues of the interleukins described in mammals (Secombes et al. 2011). *Interleukin 1β* (*il1β*) is a cytokine with pro-inflammatory functions and is involved in cell proliferation, whereas *interleukin 10* (*il10*) exhibits anti-inflammatory functions (Ángeles Esteban 2012). Both *il10* and *il1β* are of particular importance in coordinating the immune response in fish, making the study of their expression levels a good indicator of the immunity status of fish (Richard et al. 2014). The expression of *il1β* and *il10* in fish has been reported to be increased after exposure to stressors like crowding (Caipang et al. 2008) or insecticides (Ghelichpour et al. 2019), and decreased following exposure to a glyphosate-based herbicide (Richard et al. 2014). Although the effects of PAA exposure on the transcript levels of *il1β* and *il10* are yet unknown, exposure to H_2O_2, a known element of PAA-based disinfectants, was suggested to activate the inflammatory response in salmon by inducing increased expression of *il1β* in the gills (Chalmers et al. 2018), as well as in tilapia by enhancing the expression of *il1β* and inhibiting the expression of *il10* in the liver (Jia et al. 2019).

Mucins are the main components of mucous layers and are high molecular weight, filamentous and highly glycosylated glycoproteins that play a vital role in mucosal defence (Shephard 1994). Mucins are categorised into two structurally different classes: large secreted gel forming mucins and membrane-bound mucins (Sveen et al. 2017). Previously thought to simply shield and lubricate epithelial surfaces, molecular studies have revealed that mucins are also implicated in signalling pathways that enable complex and coordinated cellular responses such as cell proliferation, differentiation and adhesion, apoptosis, immune response, bacterial adhesion / inhibition, and secretion of specific cellular products, making them promising biomarkers to distinguish between normal states and inflammatory processes (Pérez-Sánchez et al. 2013).

In humans, oxidative stress is suggested to upregulate the production and secretion of mucin glycoproteins in airway mucus, namely MUC5AC and MUC5B (Fischer et al. 2015). In fish however, knowledge regarding the mucin-encoding genes remains scarce. Sveen et al. (2017) identified seven mucin genes in the Atlantic salmon genome based on annotation, transcription, phylogeny and domain structure, and found that the tissue-specific transcription pattern of *muc5* and *muc2* families are comparable to those found in other species. In particular, the genes *mucin5ac-like* (*muc5ac*) and *mucin5b-like* (*muc5b*) were found to be mainly transcribed in the skin, whereas the gene *mucin2-like* (*muc2*) was mainly transcribed in the intestine. Moreover, the authors reported that mucin expression was affected by the

presence of both an acute and a chronic stressor. Handling stress led to increased expression of some of the mucin genes in the gill, and decreased expression in the skin and intestine. Following intensive rearing and confinement stress, transcription of mucin genes in the skin was increased.

Considering the active involvement of interleukins and mucins in the fish immune response and their sensitivity to stressful stimuli, alterations on the transcriptional levels can be studied as valuable biomarkers for the stress and immune status of fish.

2 Objectives

In order to establish the potential of PAA as an effective and safe disinfectant for salmon produced in RAS, the experiment was conducted based on the following objectives:

1) To assess the magnitude of impacts on the physiology and immunology of mucosal barrier functions in salmon periodically exposed to PAA;
2) To determine whether the physiological responses of salmon exhibit habituation to repeated PAA exposure;
3) To verify how PAA disinfection affects the bacterial activity of the biofilter media and in the water-phase;
4) To investigate if periodic application of PAA disturbs the overall performance and ability of salmon to assemble a normal physiological response in the presence of a secondary stressor.

3 Materials and Methods

3.1 Ethics Statement

All fish handling procedures complied with the Guidelines of the European Union (Directive 2010/63/EU) and the trial was granted approval by the Norwegian Food Safety Authority (FOTS ID 20831).

3.2 Fish Husbandry

The trial was performed at the Nofima Centre for Recirculation in Aquaculture (NCRA) in Sunndalsøra, Norway (Fig. 1). The fish used in the trial were hatched and smoltified at NCRA, according to the station's standard protocol, as earlier described (Ytrestøyl et al. 2020). Seven hundred and thirty-five (735) smolts with an average weight of 90 g were transferred to each of the 4 x 3.2 m^3 octagonal tanks in a Recirculating Aquaculture System (RAS).The initial density in the tank was *ca* 20.6 kg/m^3. The water flow rate in the tank was set at 100 L/min. Fish were allowed to acclimatize for three weeks before the start of peracetic acid exposure under the following rearing conditions: salinity at 11.6 ± 0.5 ‰, temperature at 12.8 ± 0.6 °C, pH at 7.5, dissolved oxygen > 90 % saturation and photoperiod set at 24h light : 0h dark. During the trial, fish were fed daily with a commercial diet (Nutra Olympic 3 mm, Skretting, Averøy, Norway) administered through a belt feeder, 24h a day.

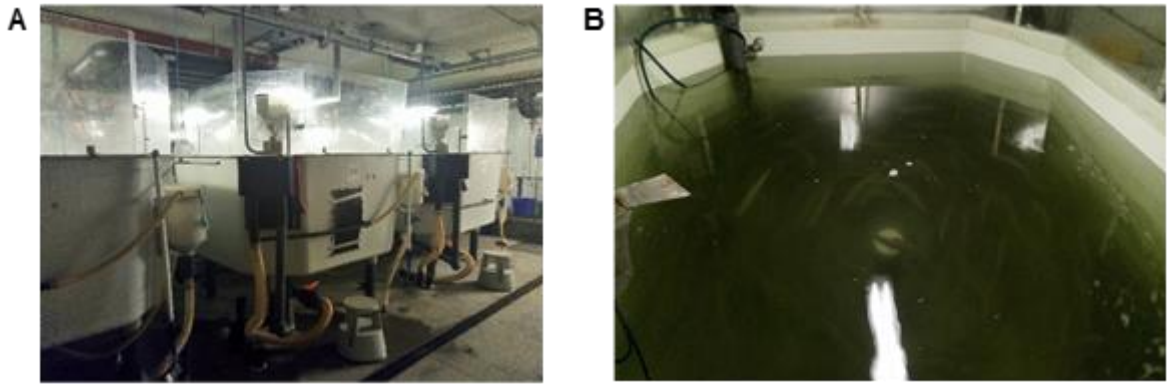

Figure 5 - Experimental facilities. Experimental rearing tanks are 3.2 m3 (A) and included filtration and control of the water parameters (B).

3.3 Peracetic acid exposure

A peracetic acid-based disinfectant (Perfectoxid, PAA), a stabilized PAA solution (1-5% v/v), was supplied by Novadan ApS (Kolding, Denmark). Following a 3-week acclimation period, PAA was applied to each tank at a nominal concentration of 1 mg/L every 3 days for a period of 6 weeks, making a total of 15 administrations during the experiment. This protocol was based on a previous experiment conducted in rainbow trout, a closely related species (Liu et al. 2020). The disinfectant was added at 4 different locations in the tank to ensure uniform

distribution and between 09:00-10:00 am to avoid temporal effects of PAA (Lazado and Voldvik 2020). The predicted exponential decay of PAA in brackish water is around 0.020-0.030 h^{-1} (Pedersen and Lazado 2020). Fish were visually monitored every day and during applications of PAA to detect any behavioural changes.

3.4 Sampling

3.4.1 Sampling schedule

During the trial, three extensive tissue samplings were performed to assess the effects of periodic PAA exposure on fish. These took place 4 days before peracetic acid exposure, on day 22 of periodic exposure and day 45 of periodic exposure. Fish were starved for 24 hours prior to sample collection. Ten fish were randomly dip-netted from each tank (N=40), sedated with metomidate ($C_{13}H_{14}N_2O_2$) at 5 mg/L, length and weight of the fish were recorded, and blood was withdrawn for plasma collection. Afterwards, the fish were humanely euthanized with an overdose (250 mg/L) of Tricaine methanesulfonate "MS-222" ($C_{10}H_{15}NO_5S$). Skin, gills, olfactory rosette, and liver were sampled both for histology and gene expression analysis.

To profile the blood chemistry (i.e. cortisol, lactate, glucose, and total antioxidant capacity) during the early phase of PAA exposure, a series of blood collection was performed within the first two weeks of administration. Five fish were randomly netted out from each of the 4 tanks (N=20) and were sedated with metomidate. Afterwards, blood was withdrawn for plasma collection and the fish were humanely euthanized thereafter with MS222. To prevent effects of the circadian rhythm in these parameters, all blood samples were collected at the same period of each day (12:00 am -13:00 pm).

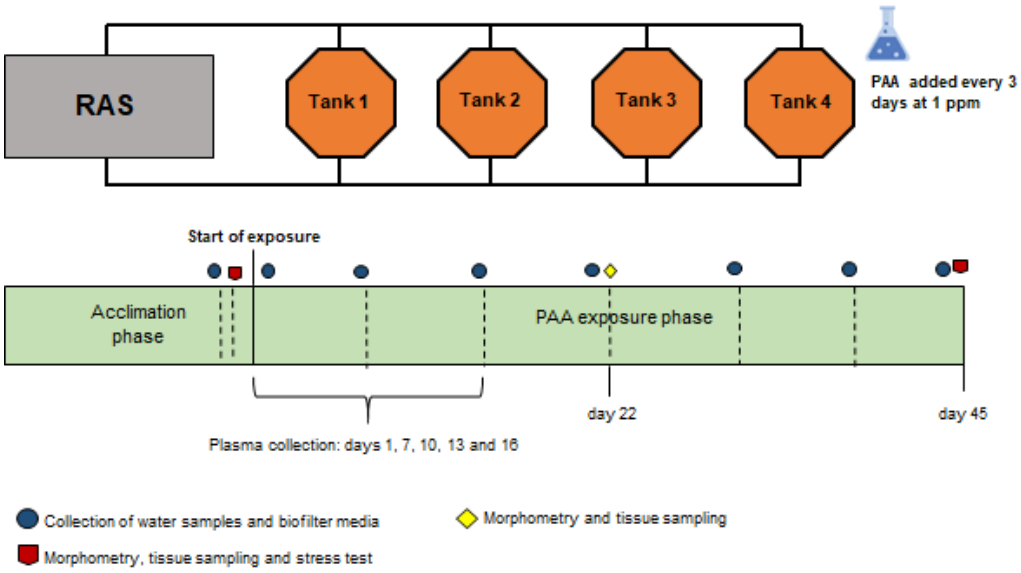

Figure 6 - The Illustration represents the Recirculation system and the sampling schedule of the experiment.

3.4.2 Blood Sampling

Blood was sampled from the caudal artery using a 4 mL lithium heparinised vacutainer (BD, Plymouth, United Kingdom). To access the caudal artery, the needle was introduced a few millimetres below the lateral line, close to the anal fin, until the spine was reached. Vacuum was created by puncturing the vacutainer with the other end of the needle to extract blood from the caudal artery (Fig. 7).

Plasma was separated by centrifugation for 10 min at 5200 rpm (Heraeus Labofuge 200, Thermo Scientific, Massachusetts, USA), transferred to 1.5mL Eppendorf tubes and stored at -80 ºC until analysis.

Figure 7 - Picture shows the blood withdrawal technique.

3.4.3 Tissue Sampling

After the length and weight of the fish were recorded, and blood was withdrawn, a section of the skin, gills, olfactory rosette and liver was dissected for histology and gene expression analysis using a scalpel equipped with a 23 blade, a rat teeth tweezers and a sharp-blunt straight scissors. Dissection of the tissues was performed on a plastic cutting board, which was disinfected with 70% ethanol after each fish.

Skin was collected by dissecting a rectangular portion (ca 2 cm x 1.5 cm) on the left side of the fish, above the lateral line and half centimetre below the dorsal fin (Fig. 8). Approximately 1/3 of the dissected portion was separated and the attached muscle tissue removed before suspending the skin tissue in RNAlater (Ambion ©, Connecticut, USA), while the other 2/3 were placed in 10% neutral buffered formalin (BiopSafe®-Biopsy Container 60mL, Stenløse, Denmark) for histological processing.

The second gill arch was collected from the left side by gently opening the operculum and cutting the whole gill arch. A small portion of the ventral part of the gill arch was placed in RNAlater, whilst the remaining part of the second gill arch was placed in formalin for histological processing. To collect the olfactory rosette, the snout of the fish was cut off cranially

to the eye and was divided in half according to the sagittal plane. One of the halves was dissected, the nostril and surrounding collagenous tissues were removed to expose the olfactory rosette, and thereafter placed in RNAlater. The other half (including the nostril) was placed in formalin. The liver was collected by sectioning the middle line of the ventral aspect of the fish, from the caudal edge of the pelvic fins to the cranial edge of the thoracic fins, and gently pulling the liver out to break its ligaments. A small portion of the liver was placed in RNAlater.

To allow penetration and ensure proper preservation of the tissues, the formalin and RNAlater containers were kept at room temperature overnight and then stored at 4 ºC and -80 ºC, respectively, until further analysis.

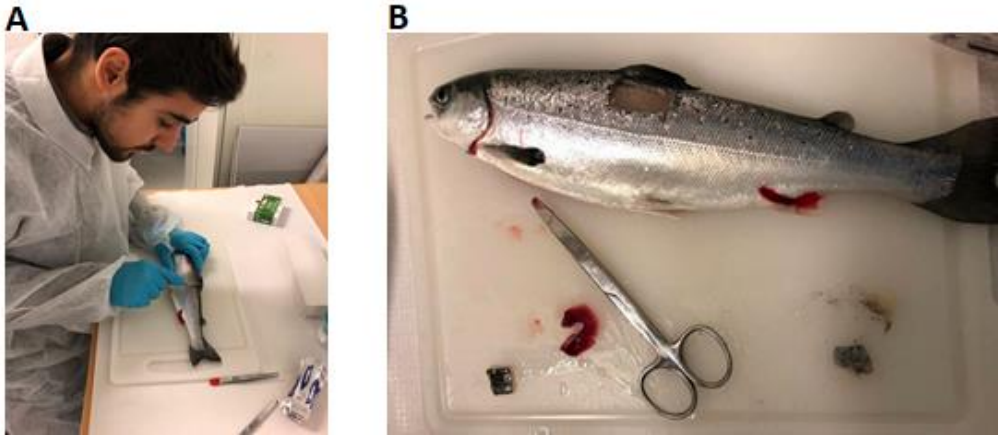

Figure 8 - Tissue sampling for histology and gene expression analysis (A; B): the site of skin sectioning and the second gill arch can be seen.

3.5 Stress test

In order to compare the ability of the pre-exposed and PAA exposed fish to mount a physiological stress response to a secondary stressor, a stress test composed of handling and confinement challenges was performed 4 days before the first PAA application (pre-exposure response) and at day 45 following the start of periodic exposure (post-exposure response). Fish were starved for 24 hours prior to the test. Before the stress test was performed, 10 fish were netted out from each tank (N = 40), sedated with metomidate (5 mg/L), blood was collected from the caudal artery by a heparinised vacutainer and fish were then euthanized with MS-222 (250 mg/L). This group of fish served as the pre-stress fish or T0. The handling-confinement stress protocol was as follows: 20 fish per tank (N = 80) were randomly dip-netted, exposed to air for 15 seconds, confined in a bucket for 5 minutes to achieve a density of 230 kg/m^3 and thereafter transferred to a recovery tank with aeration. Each experimental tank had its own corresponding recovery tank. Post-stress blood collection was performed at 1 (T1) and 3 (T3) hours after the stress test with a similar collection protocol described for T0. Ten (10) fish were taken from each recovery tank at each post-stress

sampling. Plasma was separated from the blood by centrifugation for 10 min at 5200 rpm, and thereafter stored at -80 °C until analysis.

3.6 Determination of plasma cortisol concentration

The plasma cortisol concentration was determined using a solid-phase ELISA kit (DEH3388; Demeditec Diagnostics GmbH, Kiel, Germany). The kit follows the principle of competitive binding, where an anti-cortisol antibody coats the microtiter wells. An uncertain quantity of cortisol present in each sample competes with a cortisol-horseradish peroxidase conjugate for binding with the antibody. Following incubation, wash of the unbound conjugate is performed. After adding the substrate solution, the intensity of colour developed by the bound peroxidase conjugate is inversely proportional to the concentration of cortisol present in the sample. ELISA was performed according to the manufacturer's instructions (Appendix I).

3.7 Determination of plasma glucose concentration

The plasma glucose concentration was determined using a Colorimetric Detection kit (K039-H1; Arbor Assays, Michigan, USA) for the quantitative determination of glucose in serum, plasma, urine and buffers (species independent). To generate a standard curve for the assay, a β-D-glucose standard is given. All samples must be read using the standard curve. Samples are mixed with horseradish peroxidase (HRP) and the colorimetric substrate, and the reaction is induced by the addition of glucose oxidase (GOD). The reaction is then incubated at room temperature for 30 minutes. H_2O_2 is produced by the reaction of glucose oxidase and glucose, and in the presence of HRP, H_2O_2 reacts with the substrate to convert it into a pink-coloured product. Increasing amounts of glucose trigger a linear increase in colour. The test was conducted according to the manufacturer's instructions (Appendix II).

3.8 Determination of plasma lactate concentration

The determination of plasmatic lactate concentration was performed by staff at NRCA employing the ABX Pentra Lactic Acid kit (A11A0172, HORIBA ABX, Montpellier, France) and OD was determined using Pentra C400 (HORIBA ABX, Montpellier, France). The test is based on the release of hydrogen peroxide induced by lactate oxidase, and the subsequent reaction with 4-aminoantipyrine and ESPAS to form a coloured complex in the presence of peroxidase. Colour intensity of the sample is proportional to a pre-known amount of lactate.

3.9 Determination of total antioxidant capacity in plasma

The determination of total antioxidant capacity levels was carried out by staff using a colorimetric kit (MAK187; Sigma-Aldrich, Missouri, USA).The kit is suitable for the detection of

small molecule and protein antioxidants in cell and tissue lysates, media, or in biological fluids such as plasma, serum and urine. The level of antioxidant capacity is expressed relative to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E that works as an antioxidant standard. The kit has been earlier applied to salmon plasma (Soleng et al. 2019).

3.10 Histological processing

The gill, skin and olfactory rosette samples preserved in formalin were placed into embedding cassettes. Before tissue processing, the skin and olfactory rosette were subjected to decalcification by suspending them in 10% Titriplex 3X (Sigma Aldrich) and incubated for two days with constant shaking. All samples were then kept in 70% ethanol before they were transferred to an automated tissue processor (TP1020, Leica Biosystems, Nussloch, Germany) for dehydration, clearing and paraffin infiltration, performed according to Table 1.

Table 1 - Protocol for the processing of the tissues.

step	solution	time
1	PBS	1h
2	50% EtOH	1h
3	70% EtOH	1h
4	96% EtOH	1h
5	100% EtOH	30 min
6	100% EtOH	30 min
7	100% EtOH	30 min
8	Xylen	30 min
9	Xylen	30 min
10	Xylen	30 min
11	Paraffin	1h
12	Paraffin	2h

After embedding in paraffin using a heated paraffin embedding module (Leica EG1150H, Leica Biosystems, Nussloch, Germany), samples were cut to 5 µm sections with a rotatory microtome (Leica RM2165, Leica Biosystems, Nussloch, Germany), placed onto microscope slides (Surgipath, Leica Biosystems, Illinois, USA), heat-fixed at 60 ºC overnight, dehydrated and stained using an automated stainer (ST5010, Leica Biosystems, Nussloch, Germany) with Periodic Acid Schiff-Alcian Blue (AB-PAS) according to protocol provided in Table 2. Photographs of the slides were taken using a digital slide scanner (Aperio CS2, Leica Biosystems, Illinois, USA) and analysed thereafter using Aperio Image Scope – Pathology Slide Viewing Software (version 12.4.3; Leica Biosystems, California, USA).

Table 2 - Protocol for Periodic Acid Schiff-Alcian Blue (AB-PAS) staining.

step	solution	time
1	Alcian Blue	15 min
2	Wash	3 min
3	Periodic Acid	10 min
4	Wash	3 min
5	Schiffs	15 min
6	Wash	10 min
7	Haematoxylin	30 sec
8	Wash	3 min

step	Solution	time
9	Hexamine	1 min
10	Wash	3 min
11	70% EtOH	1 min
12	96% EtOH	1 min
13	100% EtOH	1 min
14	100% EtOH	1 min
15	Xylen	1 min
16	Xylen	1 min

3.11 Histological assessment

3.11.1 Histological assessment of the gills

For the morphometric analysis of the gills, 8 fields from the whole gill arch section were randomly selected, where each field contained 40 secondary lamellae, accounting for a total of 320 secondary lamellae investigated for each fish. Mucous cells were quantified both at the primary and secondary lamellae and further differentiated as either acidic (bright blue) or neutral (magenta) mucous cells. Lamellar length (measured from base to the tip) and interlamellar space (measured from the base of one lamella to another) were measured. Moreover, a descriptive histopathological analysis was performed for each sample. Lamellar clubbing, epithelial lifting, hyperplasia, hypertrophy, lamellar fusion, and necrosis were documented. Lamellae showing none of the mentioned lesions were defined as “normal”.

3.11.2 Histological assessment of the skin

The measurement was carried out in 3 randomly selected regions of *ca* 500 µm in distance per region. In each region, epidermal mucous cells were quantified and differentiated as acidic or neutral mucous cells. Epidermal and dermal thickness were also measured in 5 different locations within the selected region.

The microscopic general appearance of the epidermis and the quality of the epithelial surface were characterized using the semi-quantitative skin health scoring system earlier described (Sveen et al. 2018; Lazado, Haddeland, et al. 2020).

Table 3 - Semi-quantitative skin health scoring system for the microscopic epidermal general appearance and epithelial surface quality. Adapted from Sveen et al. (2018) and Lazado, Haddeland et al. (2020).

Epidermis	Score	Description
General Appearance	0	Even epidermis all over
	1	Uneven epidermis
	2	Parts of the epidermis is missing
	3	Most of the epidermis is missing
Surface	0	Smooth surface
	1	Signs of rough cells at the surface
	2	Clear signs of rough cells, <50% of the surface affected
	3	All cells lining the outer part of the epidermis appear rough

3.11.3 Histological assessment of the olfactory rosette

For the olfactory rosette, measurements were taken in 3 randomly selected olfactory lamellae per fish. Thickness of the olfactory epithelium and lamina propria were systematically measured in 5 distinct locations within the mid-region of the olfactory lamellae so that uniformity was assured. Given the high density of mucous cells per unit area, it was difficult to perform an impartial and exact count of the number of mucous cells. Thus, a descriptive assessment from two evaluators was performed instead.

3.12 Isolation of Ribonucleic Acid

Total ribonucleic acid was isolated from the gills, skin, olfactory rosette, and liver using Quick-RNA™ Microprep Kit (R1051, Zymo Research, California, USA). The Quick-RNA™ Microprep Kit allows a fast and reliable isolation of DNA-free RNA from an extensive range of cell and tissue samples. The kit relies on a buffer system with Clean-Spin column technology to isolate high quality total RNA in a short period of time.

Isolation of RNA was achieved by (1) sample lysis/homogenization, (2) sample clearing and (3) RNA purification, and was executed according to the manufacturer's instructions with adaptations (Appendix III).

3.13 Determination of RNA concentration and quality

After RNA isolation, the concentration and quality were determined at 260 nm and 280 nm using the NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). The ratio of optical densities measured at 260 nm, specific for nucleic acids, and 280 nm, specific for proteins,

was calculated as it gives information on the quality of the RNA sample. Only samples with a ratio of 1.9 - 2.1 were kept, since it indicates good RNA quality without protein contamination (Manchester 1996).

3.14 cDNA synthesis by reverse transcription

cDNA was synthesized by reverse transcription of 500 ng total RNA in a 20 µL reaction using Taqman™ Reverse Transcription Kit (Applied Biosystems, ThermoFisher, Massachusetts, USA) with random hexamers as reaction primers. The 20 µL reaction was set up containing 9.6 µL 500 ng template RNA, 2 µL 10X RT Buffer, 1.4 µL 25mM $MgCl_2$, 4 µL 10mM dNTP mix, 1 µL RNase Inhibitor, 1 µL MultiScribe™ Reverse Transcriptase and 1 µL Random Hexamers. Thermocycling was performed using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, California, USA) and parameters were as follows: 25 ºC for 10 min, 37ºC for 30 min and 95ºC for 5 min.

3.15 Real-time quantitative polymerase chain reaction

Gene expression analysis by RT-qPCR was performed in three different stages. To start, a five-step standard curve of 2-fold dilution series was prepared from pooled cDNA of the 3 major sampling points (before exposure, and at 22 and 45 days post-exposure) to calculate the amplification efficiency. Afterwards, three housekeeping genes *Elongation factor alpha-1*, *Acidic ribosomal protein,* and *B-actin* (***ef1α***, *arp* and *actb*) were tested for their suitability for normalization of the expression data in the gills, skin, olfactory rosette and liver. Finally, gene expression analysis of the target genes *gpx*, *gr*, *gsta*, *mnsod*, *cu/znsod*, *cat*, *il1β*, *il10*, *hsp70*, *hsp90, muc5ac*, *muc5b* and *muc2* was carried out and transcript levels were expressed as relative expression after normalization using the geometric mean of reference genes ***ef1α*** and *actb* (Nagasawa et al. 2012).

RT-qPCR was performed using QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, USA) in a 384-well plate. PCR master mix was prepared on ice and consisted of 5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) and 0.5 µL 10 µM of each forward/reverse primer (Invitrogen, USA). Primer pairs are listed in Table 4.

The 384-well plates were placed on ice while each well was pipetted with 4 µL 1:10 diluted cDNA, followed by the addition of 6 µL of the master mix, meaning each PCR assay had a final volume of 10 µL. Each assay was pipetted and ran in duplicate. The 384-well plates were then briefly centrifuged and transferred to the QuantStudio™ 5 system with cycling parameters as follows: pre-incubation at 95ºC for 20 seconds, amplification with 40 cycles at 95 ºC for 1 second and 60ºC for 20 seconds, and a dissociation stage of 95ºC for 1 second, 60ºC for 20 seconds and 95ºC for 1 second.

Table 4 - Primer pairs used in the present study.

Gene name	Abbreviation	Sequences (5' → 3')	Reference
Glutathione peroxidase	*gpx*	F: GATTCGTTCCAAACTTCCTGCTA R: GCTCCCAGAACAGCCTGTTG	(Solberg et al. 2012)
Glutathione reductase	*gr*	F: CCAGTGATGGCTTTTTTGAACTT R: CCGGCCCCCACTATGAC	(Solberg et al. 2012)
Glutathione S-transferase	*gsta*	F: AGGGCACAAGTCTAAAGAAGTC R: GTCTCCGTGTTTGAAAGCAG	(Lazado and Voldvik 2020)
Manganese superoxide dismutase	*mnsod*	F: GTTTCTCTCCAGCCTGCTCTAAG R: CCGCTCTCCTTGTCGAAGC	(Solberg et al. 2012)
Copper/Zinc superoxide dismutase	*cu/znsod*	F: CCACGTCCATGCCTTTGG R: TCAGCTGCTGCAGTCACGTT	(Solberg et al. 2012)
Catalase	*cat*	F: GGGCAACTGGGACCTTACTG R: GCATGGCGTCCCTGATAAA	(Olsvik et al. 2011)
interleukin 1β	*il1b*	F: AGGACAAGGACCTGCTCAACT R: CCGACTCCAACTCCAACACTA	(Ingerslev et al. 2009)
interleukin 10	*il10*	F: GGGTGTCACGCTATGGACAG R: TGTTTCCGATGGAGTCGATG	(Ingerslev et al. 2009)
Heat shock protein 70	*hsp70*	F: CCCCTGTCCCTGGGTATTG R: CACCAGGCTGGTTGTCTGAGT	(Solberg et al. 2012)
Heat shock protein 90	*hsp90*	F: CCACCATGGGCTACATGATG R: CCTTCACCGCCTTGTCATTC	(Olsvik et al. 2007)
Mucin 5ac-like	*muc5ac*	F: GACCTGCTCTGTGGAAGGAG R: AGCACGGTGAATTCAGTTCC	(Sveen et al. 2017)
Mucin 5b-like	*muc5b*	F: ATTAAGAGCGATGTCTTCACAGC R: AAGCACATGAGTCTCTCACACAA	(Sveen et al. 2017)
Mucin 2-like	*muc2*	F: GAGTGGGCTCTCAGATCCAG R: GATGATGCGGACGGTAGTTT	(Sveen et al. 2017)
Elongation factor alpha 1	*ef1a*	F: GAATCGGCTATGCCTGGTGAC R: GGATGATGACCTGAGCGGTG	(Garcia de la serrana and Johnston 2013)
B-actin	*actb*	F: CCAAAGCCAACAGGGAGAA R: AGGGACAACACTGCCTGGAT	(Sanden and Olsvik 2009)

3.16 Determination of bacterial activity in the water phase

In order to assess the impact of periodic disinfection with PAA on the bacterial dynamics in the water phase, water samples were collected from each of the four tanks on a weekly basis and bacterial activity was quantified using the Bactiquant® Water test-kit (Mycometer, Copenhagen, Denmark). The kit is a fast and reliable tool for the detection and quantification of total bacteria in water samples, targeting a broad spectrum of bacteria, including gram positive, gram negative, aerobic and anaerobic bacteria, regardless of chemical and physical properties of the water samples. The bacteria are quantified based on enzymatic activity,

measured in a highly sensitive fluorometer, with the magnitude of the fluorescence signal being directly proportional to the bacterial activity in the sample. The test was performed according to the manufacturer's protocol (Appendix IV).

3.17 Determination of the bacterial activity in the media/surface of the Moving Bed Biofilter Reactor

With the intention of understanding the effects of periodic PAA disinfection on the bacterial activity in the Moving Bed Biofilter Reactor (MBBR), a total of five media/chips was collected from the three compartments of the MBBR on a weekly basis and analysed using the Bactiquant® Surface test-kit (Mycometer, Copenhagen, Denmark). The kit allows for the determination of total bacterial levels present on surfaces and is based on the detection of a fluorescent compound, formed by the reaction of a bacterial hydrolytic enzyme with a provided enzyme substrate. The test was performed following the manufacturer's guidelines (Appendix V).

3.18 Data handling and treatment

A Shapiro-Wilk test was used to evaluate the normal distribution and a F-test to check for the equal variance of the data from plasma stress indicators, total antioxidant capacity, gene expression analysis, histological assessment and bacterial activity. A one-way analysis of variance (ANOVA) was used to test for differences between exposure periods, followed by Tukey's multiple comparison test when significant differences were observed. The Holm-Sidak test was used to identify pairwise differences. For the data of epidermal general appearance and surface quality, a Fisher's Exact test was performed. Statistical tests were executed using R studio (version 1.2.5019) and the level of significance was set at $p<0.05$, except when the Holm-Sidak test was performed, for which the level of significance was set at $p<0.025$. Values are expressed as mean ± SE.

4 Results

4.1 Production Performance

No treatment-related mortality was recorded during the trial. In addition, daily visual inspection revealed no significant changes in the feeding behaviour. This was supported by the average weight of the fish at the end of the trial (*ca* 290 g) which was close to the projected weight of 320 g extrapolated from water temperature and daily feeding ration.

4.2 Stress Parameters in the plasma of PAA-exposed salmon

No significant temporal changes were recorded in the plasma cortisol levels during the 3 major sampling points of the experiment ($p=0.56$; Fig. 9A). Similarly, there were no significant changes in the cortisol levels during the first 2 weeks of exposure ($p=0.03$; Fig. 9B). Plasma glucose levels exhibited a 26 % and 20 % decrease at days 22 ($p=0.01$) and 45 ($p=0.049$) of periodic exposure, respectively, relative to the pre-exposure level (Fig. 9C). During the first 2 weeks of exposure, glucose levels remained stable, with no significant differences noted (Fig. 9D). Plasma lactate levels at day 22 and 45 post-exposure showed no significant differences ($p=0.004$) compared with the level before PAA administration. Nonetheless, a reduction of 34 % in the plasma lactate levels was observed from day 22 to 45 post-exposure ($p=0.004$; Fig. 9E). An increasing trend was noted in the plasma lactate levels during the first 2 weeks of exposure (Fig. 9F). The registered levels from day 10 onwards differed significantly ($p<0.001$) from the level at day 1 of PAA exposure, with an increment of 1-fold identified at day 13 of periodic exposure.

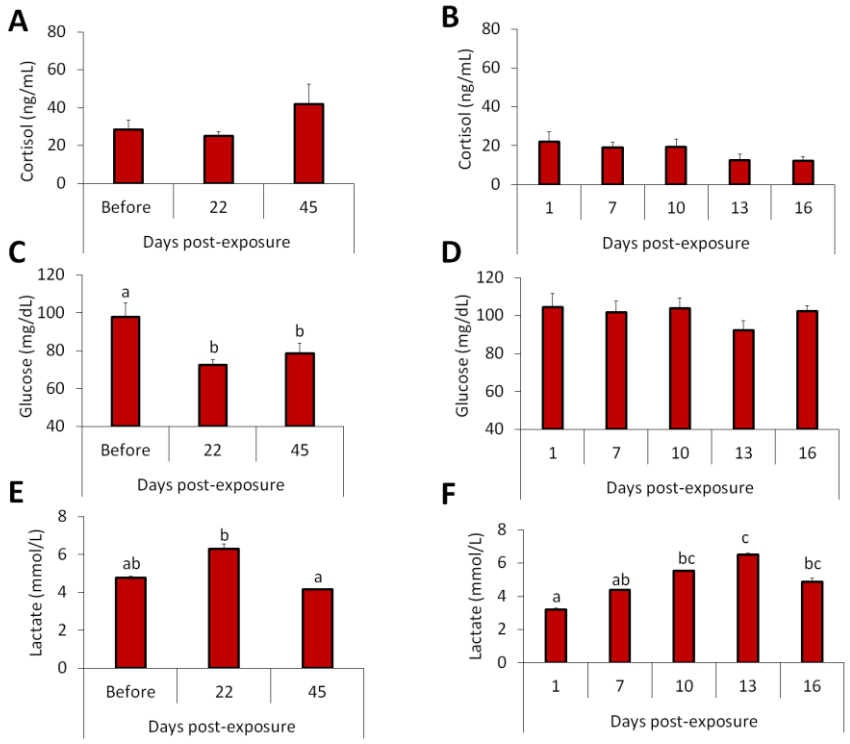

Figure 9 - Plasma levels of key stress response indicators (cortisol, glucose and lactate) in Atlantic salmon periodically exposed to PAA. The left panel (A, C, E) shows the levels measured during the 3 major sampling points (before, and at days 22 and 45 of periodic exposure), whereas the right panel (B, D, F) shows the measured levels during the first 2 weeks of periodic application. Values presented are mean ± SE of 8 individual fish per sampling point. Different letters denote significant difference.

The total antioxidant capacity (TAC) in the plasma exhibited an increase of 1-fold at day 22 ($p=0.04$) and 1.3-fold at day 45 post-exposure ($p<0.001$), compared with the pre-exposure level (Fig. 10A). As for the first 2 weeks of PAA exposure, no significant changes ($p=0.26$; Fig. 10B) on the TAC levels were observed.

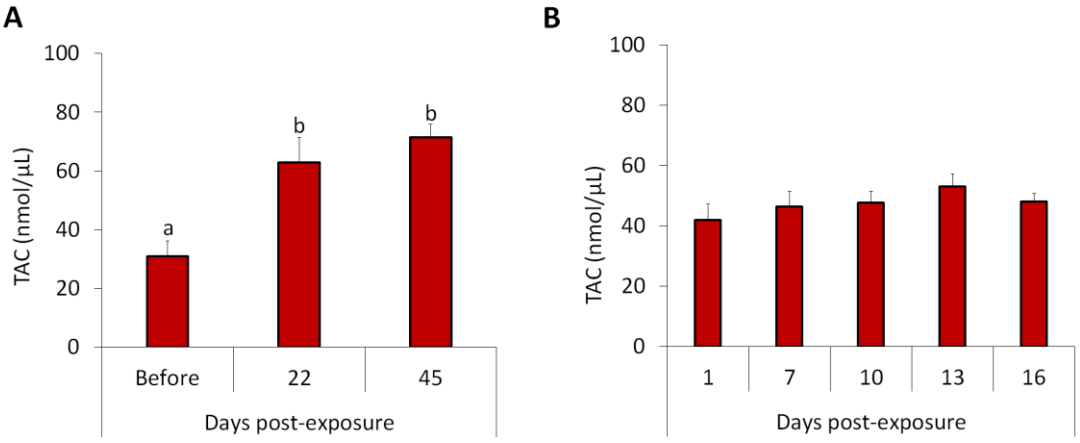

Figure 10 - Total antioxidant capacity (TAC) in the plasma of salmon collected before and 22 and 45 days after exposure (A) and collected during the first 2 weeks of periodic PAA exposure (B).

Values presented are mean ± SE of 8 individual fish per sampling point. Different letters denote significant difference.

4.3 Structural and morphometric changes in the mucosal organs

4.3.1 Skin

Key morphometric parameters of the skin such as epidermal and dermal thickness were distinctively affected by periodic exposure to PAA (Table 5) – while no significant changes ($p=0.095$) were found in the epidermal thickness, a significant dermal thickening was noted at day 45 ($p<0.001$) of periodic exposure relative to pre-exposure and day 22. No significant differences were observed neither in the number of mucous cells ($p=0.1$), nor in the proportion of acidic ($p=0.1$) and neutral mucous cells ($p=0.66$). A semi-quantitative skin health scoring approach revealed no significant differences ($p=1.0$) on the general appearance of the epidermis during the period of the trial, although all recorded cases received a score of 2 and above. Conversely, epidermal surface quality showed significant differences overtime ($p=0.005$), and cases with score 2 were more prevalent at day 45 post-exposure (Fig. 11A).

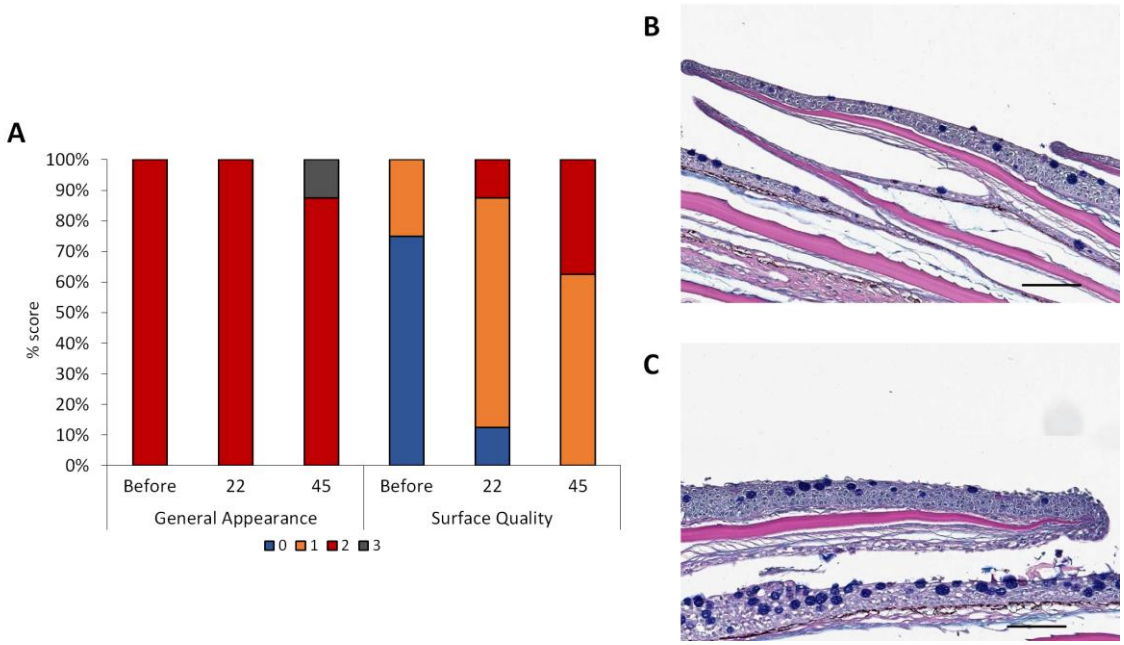

Figure 11 - Distribution of skin health scores (i.e., epidermal general appearance and epidermal surface quality) of Atlantic salmon (N=8) (A). Representative pictures of the skin showing a smooth surface of the epidermis before exposure (B) compared with clear signs of rough cells in the surface of the epidermis at 45 days of periodic exposure (C). Sections were stained with AB/PAS. Scale bar = 100µm.

Table 5 - Morphometric measurements in the skin and gills of Atlantic salmon periodically exposed to PAA. Different letters indicate significant differences between time-points. * First number indicates the total number of mucous cells, whereas the parenthesis show the ratio of acidic and neutral mucous cells, respectively. Values presented are mean ± SE of 8 individual fish per sampling point.

Tissue	Parameters	Days post-exposure		
		0	22	45
Skin	Epidermal thickness	38.5 ± 3.6 µm	39.7 ± 6.2 µm	50.6 ± 3.4 µm
	Dermal thickness	136.9 ± 5.6 µm^{a}	127.9 ± 4.8 µm^{a}	180.1 ± 7.3 µm^{b}
	Number of mucous cells*	18.8 ± 1.8 (16.8/2)	21.6 ± 3.8 (19.6/2)	27.2 ± 2.4 (24.6/2.5)
Gills	Lamellar space	31.6 ± 0.7 µm^{a}	31.8 ± 0.8 µm^{a}	26.6 ± 0.6 µm^{b}
	Lamellar length	125.3 ± 3.8 µm^{a}	140.5 ± 3 µm^{b}	160.4 ± 2.4 µm^{c}
	Number of mucous cells*	42.9 ± 4^{a} (37.4^{a}/5.5)	83 ± 6.8^{a} (76.6^{a}/6.3)	88.9 ± 10.1^{b} (84.3^{b}/4.6)

4.3.2 Gills

4.3.2.1 Morphometric assessment of the gills

Interlamellar space significantly decreased at day 45 post-exposure (Tab. 5) relative to pre-exposure ($p<0.001$) and at day 22 post-exposure ($p<0.001$). Lamellar length progressively increased during the exposure trial ($p<0.001$). The total number of mucous cells at day 45 post-exposure was significantly higher than before exposure ($p<0.001$) and at day 22 ($p<0.001$) post-exposure. The same pattern was identified for the number of acidic mucous cells (day 22, $p<0.001$; day 45, $p<0.001$), but not for the number of neutral mucous cells ($p=0.83$).

4.3.2.2 Descriptive analysis of the gills

The gill tissue presented morphological changes following periodic exposure to PAA. Gill hyperplasia, epithelial lifting (Fig. 12B), chloride cell hypertrophy (Fig. 12C), lamellar clubbing (Fig. 12D) and necrosis became more prevalent with longer periods of exposure. In the pre-exposed fish, the gills presented a normal structure. The lamellae were well differentiated and showed a normal cell morphology and structure (Fig. 12A). After 22 days of periodic exposure some changes were observed: hyperplasia, hypertrophy, epithelial lifting and lamellar clubbing were more frequently documented. Furthermore, at 45 days of periodic exposure to PAA, groups of secondary lamellae with hyperplasia, hypertrophy, epithelial lifting and occasionally necrosis of the gill epithelium were observed. Although hyperplasia was more common in distal areas of secondary lamellae, some areas showed hyperplasia in the interlamellar space and fusion of the secondary lamellae. Overall, changes in the gill epithelium became more frequent and evident after periodic exposure to PAA. After 45 days of exposure, changes were more severe compared with at 22 days post-exposure, where the lesions previously observed were more pronounced and frequent, and necrosis was sporadically found in proximal areas of the secondary lamellae.

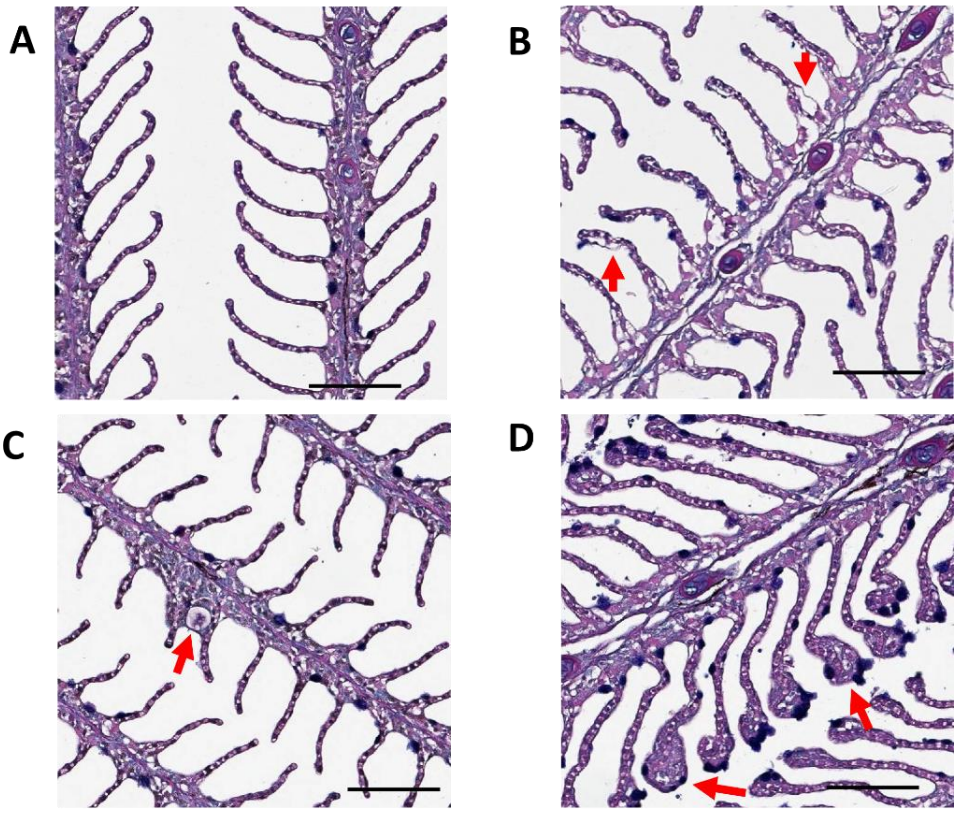

Figure 12 - Representative pictures showing normal gills from pre-exposed fish (A), and 3 of the most common pathologies identified including epithelial lifting (B), hypertrophy (C) and lamellar clubbing (D) as shown by arrowheads. Sections were stained with AB/PAS. Scale bar = 100µm.

4.3.3 Olfactory rosette

The olfactory epithelium thickness significantly increased (p=0.015) from before exposure to day 22 post-exposure (Fig. 13A). At day 45 however, the thickness of the epithelium did not significantly vary from the two previous sampling points. The lamina propria displayed a progressive thickening, where thickness at day 45 was significantly higher (p=0.03) than prior to exposure. Albeit no measurements were undertaken due to the impossibility of differentiating individual mucous cells, two independent evaluators reported a clear tendency that mucous cell coverage on the tip of the olfactory lamellae became denser after periodic disinfection with PAA (Fig. 13B-C). Moreover, mucous cells were mostly concentrated on the tip of the olfactory lamella and the walls of the nasal epithelium.

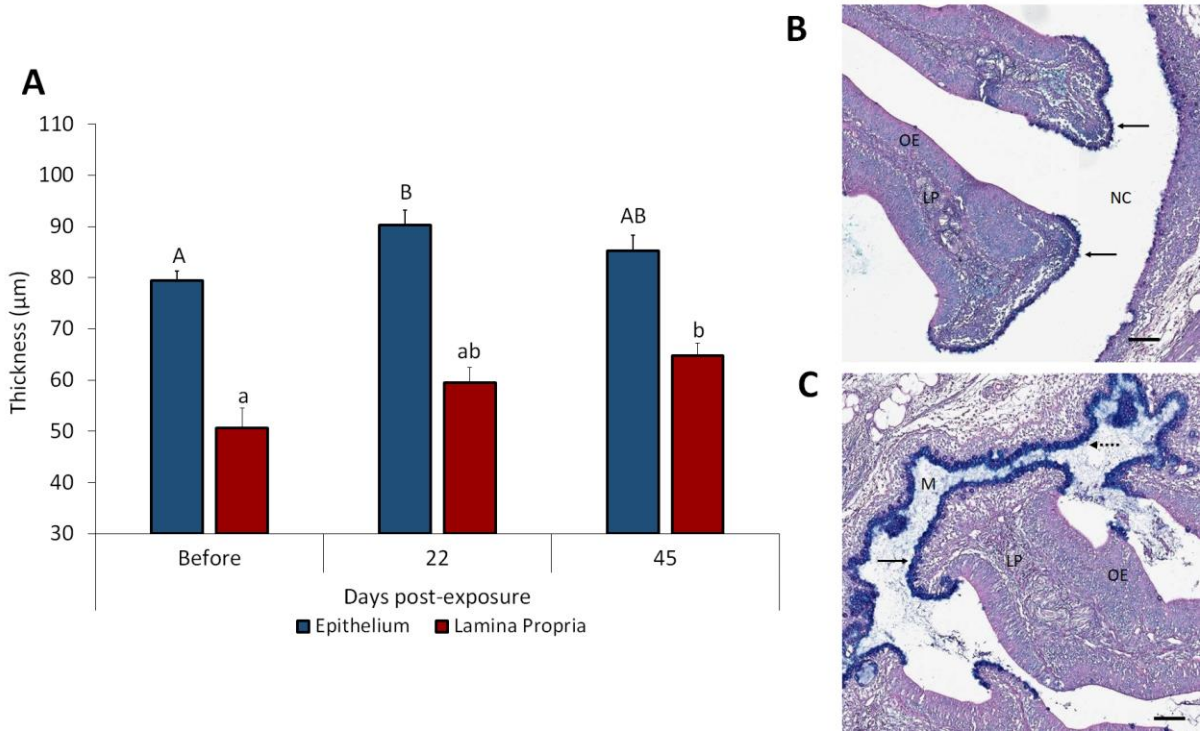

Figure 13 - Histomorphological features of the olfactory rosette of Atlantic salmon periodically exposed to PAA. Thickness of the olfactory epithelium and lamina propria (A). Values presented are mean ± SE of 8 individual fish per sampling point. Different letters denote significant difference. Uppercase letters were used for epithelial thickness and lowercase letters for thickness of the lamina propria. Representative pictures of the olfactory rosette stained with AB/PAS showing a considerable increase in the mucous cells (arrow; stained dark blue) at the

tip of the olfactory lamella in the pre-exposure group (B) compared with at day 45 (C). NC = nasal cavity; LP = lamina propria; OE = olfactory epithelium; M = mucus. Scale bar = 100µm.

4.4 Relative expression of selected immune and stress-related genes in three mucosal tissues and the liver

The transcriptional levels of 13 selected immune and stress-related genes were studied in the gills (Fig. 14A-M) and the skin (Fig. 15A-M) of salmon submitted to a periodic PAA exposure protocol. From the antioxidant-defence genes, the expression of *gr* (Fig. 14B) and *cu/znsod* (Fig. 14E) in the gills was significantly modulated by periodic exposure – the expression of *gr* at days 22 and 45 post-exposure was significantly downregulated (p=0.01; p=0.025) relative to the pre-exposure level, whereas the expression of *cu/znsod* was only significantly lower at day 22 post-exposure (p=0.02) compared with pre-exposure. In the skin, only the expression of *gr* (Fig. 15B) was modulated by periodic oxidant exposure, showing a higher transcript level at day 45 post-exposure compared with transcript levels before (p=0.007) and 22 days after PAA administration (p=0.005).

The expression of the genes *il1β* an *il10* was significantly modulated by periodic PAA exposure in the gills (Fig. 14G,H) but not in the skin (Fig. 15G,H). The expression of both genes in the gills was significantly downregulated (*il1β* p=0.01; *il10* p<0.001) at day 22 post-exposure relative to the before exposure level. A significant downregulation was also observed for *il1β* at day 45 post-exposure relative to pre-exposure level.

For the transcriptional profile of *hsp70* and *hsp90* in the gills (Fig. 14I,J) and the skin (Fig. 15I,J), only the branchial expression of *hsp90* was significantly influenced by periodic oxidant exposure, where a downregulation was noted at day 22 post-exposure (p=0.02) in relation to pre-exposure levels.

The expression of all three studied mucin genes was significantly affected by periodic PAA exposure in the two mucosal tissues (Fig. 14K,L,M, Fig.15K,L,M). The expression of *muc5ac* in the gills was significantly lower at day 45 post-exposure (p=0.024) than the level before PAA administration (Fig. 14K). *Muc5ac* in the skin displayed significantly higher transcript level at day 45 post-exposure (p=0.01) compared with at day 22 post-exposure, but not with the pre-exposure level (Fig. 15K). *Muc5b* and *muc2* exhibited a similar pattern of expression in the gills, where transcript levels were significantly lower at day 22 post-exposure (*muc5b* p=0.018; *muc2* p=0.002) than before exposure (Fig. 14L,M). Moreover, the expression of *muc5b* and *muc2* in the skin was also marked by a similar expression pattern. A significant increase in transcript levels was observed for both genes at day 45 post-exposure (*muc5b* p=0.018; *muc2* p=0.01), relative to day 22 post-exposure (Fig. 15L,M). Nevertheless, no changes were noted in relation to the pre-exposure levels.

In the olfactory rosette (Fig. 16A-M), the expression of *cat* was significantly downregulated at day 45 post-exposure (p=0.021) compared with the level before exposure, but it was not significantly different to the level at day 22 of periodic exposure (Fig. 16F). The transcriptional levels of the remaining antioxidant defence genes showed no significant variation during the experiment. The expression of *il1β* in the olfactory rosette was not significantly affected throughout the trial (Fig. 16G). Conversely, a significant downregulation of *il10* occurred at day 45 post-exposure (p=0.043), relative to the pre-exposure level (Fig. 16H). Both hsp-coding genes were significantly modulated by periodic PAA exposure (Fig. 16I,J). Decreased expression of *hsp70* (day 22 p=0.007, day 45 p=0.022) and *hsp90* (day 22 p=0.029, day 45 p=0.026) in the olfactory rosette was noted at days 22 and 45 of periodic exposure compared with levels registered before exposure. None of the mucin genes displayed signs of significantly modulated expression in the olfactory rosette following PAA administration (Fig. 16K,L,M).

Periodic PAA exposure did not significantly affect the expression of any of the selected genes in the liver (Fig. 17A-M).

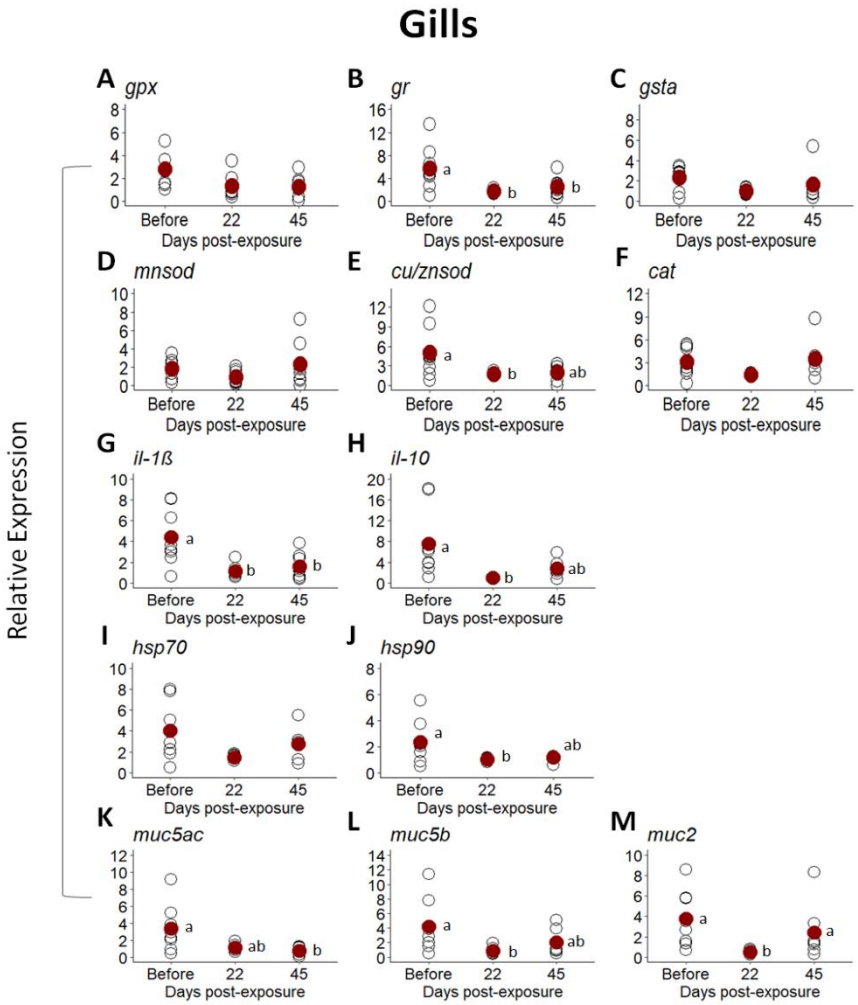

Figure 14 - Relative expression of selected genes in the gills (A→M) of Atlantic salmon periodically exposed to PAA. Each unfilled circle represents the expression of a particular gene in a single fish. The mean expression value from 8 individual fish per sampling point is denoted by the red circle. Samples were collected before and 22 and 45 days after periodic exposure. Different letters denote significant difference.

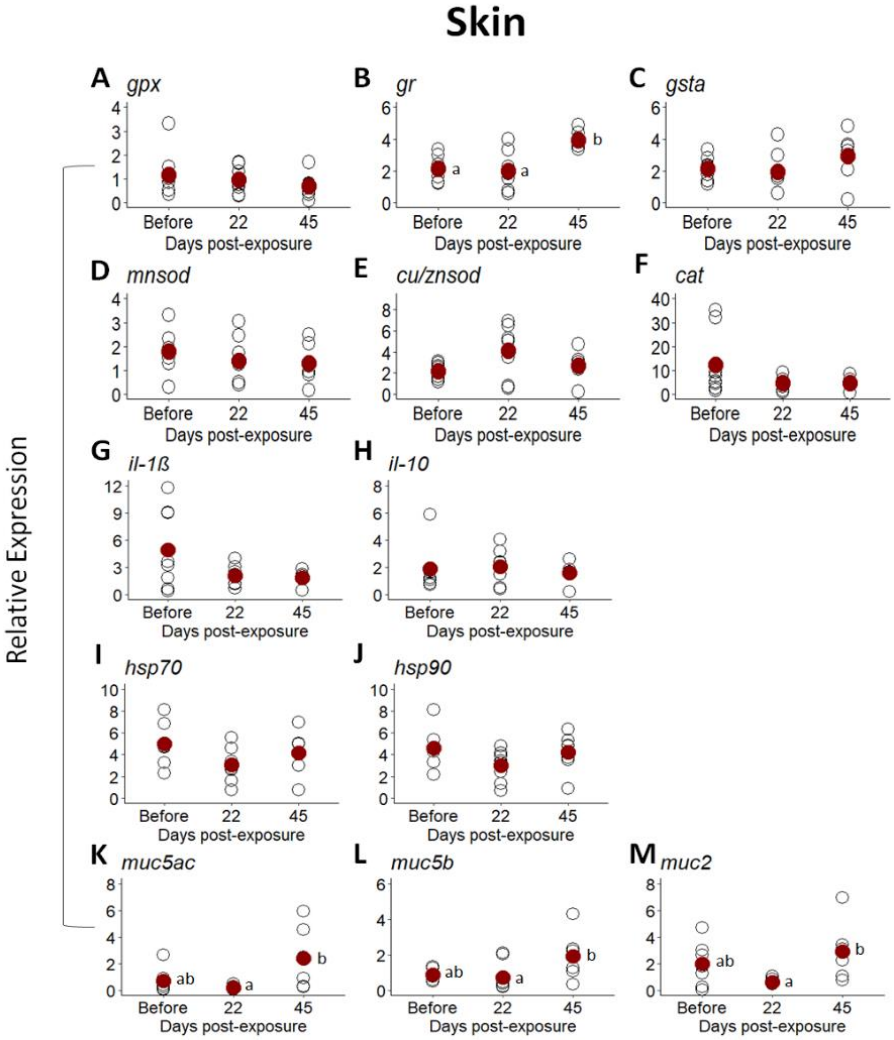

Figure 15 - Expression of selected genes in the skin (A→M) of Atlantic salmon periodically exposed to PAA. Each unfilled circle represents the expression of a particular gene in a single fish. The mean expression value from 8 individual fish per sampling point is denoted by the red circle. Samples were collected before and 22 and 45 days after periodic exposure. Different letters denote significant difference.

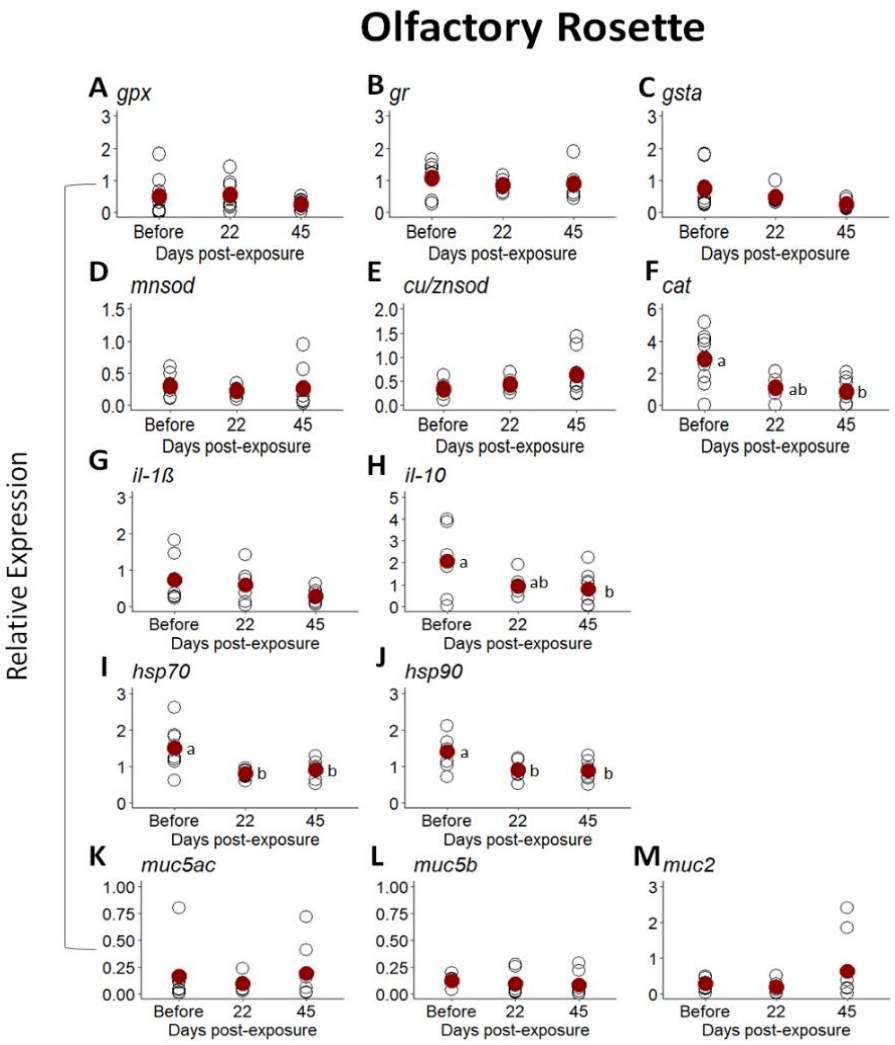

Figure 16 - Expression of selected genes in the olfactory rosette (A→M) of Atlantic salmon periodically exposed to PAA. Each unfilled circle represents the expression of a particular gene in a single fish. The mean expression value from 8 individual fish per sampling point is denoted by the red circle. Samples were collected before and 22 and 45 days after periodic exposure. Different letters denote significant difference.

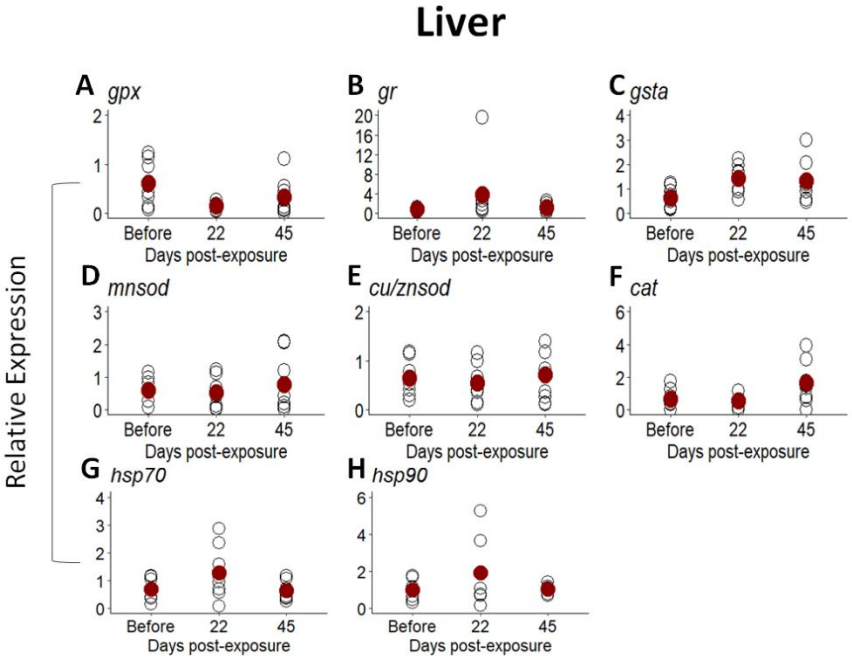

Figure 17 - Expression of selected genes in the liver (A→M) of Atlantic salmon periodically exposed to PAA. Each unfilled circle represents the expression of a particular gene in a single fish. The mean expression value from 8 individual fish per sampling point is denoted by the red circle. Samples were collected before and 22 and 45 days after periodic exposure. Different letters denote significant difference.

4.5 Physiological adaptive responses to a secondary stressor

Following the handling-confinement stress, plasma cortisol levels increased both in unexposed fish and fish periodically exposed to 1 mg/L of PAA for 45 days (Fig. 18A). Cortisol levels displayed a 5.9-fold increment ($p=0.0125$) in pre-exposed fish, while a 5.5-fold increase ($p<0.001$) was observed in the periodically exposed group 1 hour after the stress was prompted. After 3 hours, cortisol levels remained elevated relative to 0 h (unexposed group, $p=0.06$; periodically exposed group, $p<0.001$) in both groups. Comparing the two groups at a particular time-point, cortisol levels at 1 h after stress did not significantly differ between groups. On the other hand, at 3 hours after stress was induced, periodically exposed fish exhibited significantly higher ($p=0.009$) cortisol levels than pre-exposed fish.

Plasma glucose levels remained unchanged in both groups following exposure to a handling-confinement challenge (Fig. 18B). Nonetheless, plasma glucose levels in the pre-exposed group appeared to be relatively higher than in the fish periodically exposed to PAA, particularly at 3 hours after stress, where a significant difference was in fact identified ($p=0.04$).

The handling-confinement stress did not significantly alter the plasma lactate levels ($p>0.05$) neither in the pre-exposed nor the periodically exposed salmon (Fig. 18C).

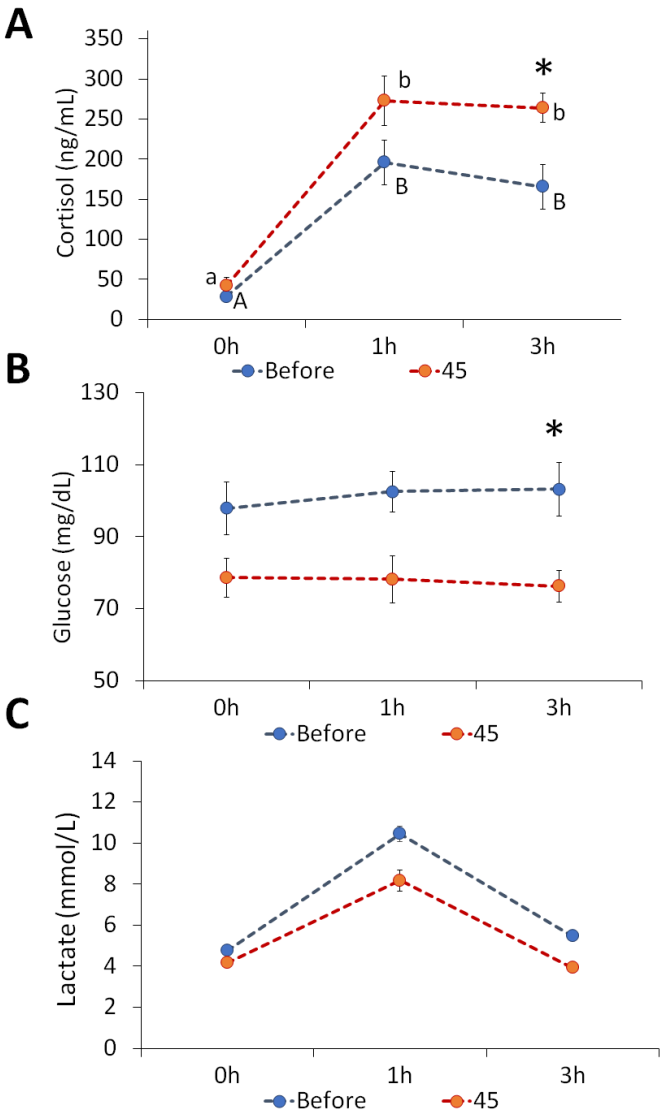

Figure 18 - Stress parameters (cortisol - A, glucose - B and lactate - C) in the plasma of Atlantic salmon subjected to handling-confinement stress. The stress test was conducted before the start of PAA exposure and at day 45 of periodic exposure. Values presented are mean ± SE of 8 individual fish per sampling point. Different letters indicate significant differences, where uppercase letters were used for the pre-exposure group and lowercase letters for the 45 days of periodic exposure group. Asterisk denotes a significant difference between the two groups at a specific time-point.

4.6 Bacterial activity in the water of the rearing tanks

Some temporal variations were observed in the level of bacterial activity in the water of the rearing tanks (Fig. 19). In particular, bacterial activity (expressed in Bactiquant Values) was significantly higher at 1 day before start of exposure ($p=0.02$; $p<0.001$) and at day 3 of periodic exposure ($p=0.02$; $p<0.001$) compared with at day 27 and day 44 of periodic exposure.

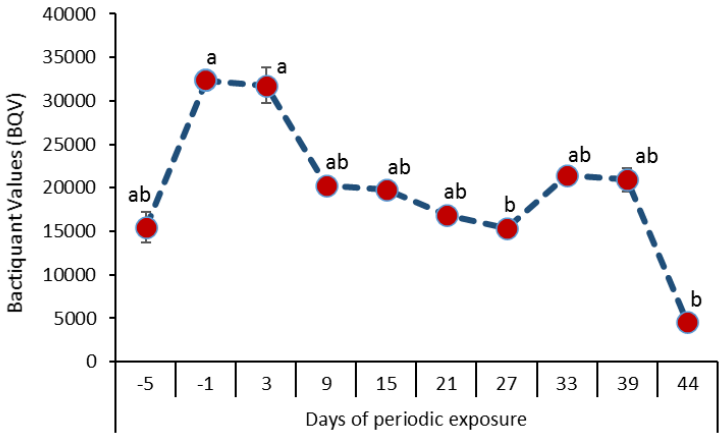

Figure 19 - Dynamics of bacterial activity in water phase at several periods from 5 days before to the end of periodic PAA administration. Values presented are mean ± SE of 4 individual rearing tanks from the same recirculating system per sampling point. Different letters denote significant difference.

4.7 Bacterial activity in the media of the MBBR

No significant changes were noted in the level of bacterial activity in the surface of the biofilter media during the period of PAA exposure. However, significantly increased values were found at day 21 ($p<0.001$) and day 44 post-exposure ($p<0.001$), relative to what was observed 5 days prior to the start of periodic PAA administration (Fig. 20).

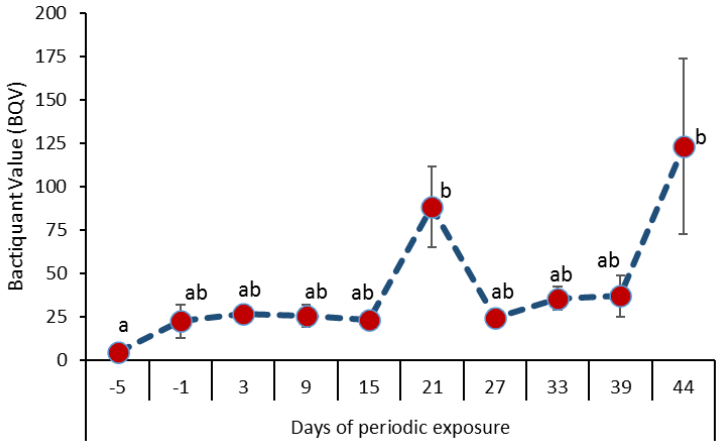

Figure 20 - Dynamics of bacterial activity in the media of the MBBR from 5 days before to the end of periodic PAA administration. Values presented are mean ± SE of 5 individual media per sampling point. Different letters denote significant difference.

5 Discussion

The adoption of PAA as a routine disinfectant in aquaculture production must be supported by a holistic understanding of how it influences the health and welfare of fish. To the best of the author's knowledge, this is the first study reporting the physiological and histostructural consequences of periodic PAA exposure in Atlantic salmon post-smolts reared in brackish water RAS. The systemic and mucosal changes suggest that the fish mobilised a network of physiological defences to adapt to an oxidant-rich environment. No treatment-related mortality and no changes, namely in the feeding behaviour were documented, indicating that welfare and production performance were not affected.

5.1 Physiological stress responses of Atlantic salmon periodically exposed to PAA

Increase in plasmatic cortisol is an adaptive response to stressors in fish (Wendelaar Bonga 1997; Barton 2002). As previously mentioned, elevated cortisol levels were reported in rainbow trout and carp after exposure to PAA, followed by negligible change after repeated pulse exposures, suggesting a form of habituation to the oxidant (Liu, Straus, et al. 2017; Liu, Pedersen, et al. 2017). In another study (Liu et al. 2018), mirror carp periodically exposed to PAA at 1 mg/L showed a significant and persistent reduction of cortisol levels compared to the unexposed fish, where the authors implied a stress-protection role of PAA. Nonetheless, this reduction of plasmatic cortisol did not reach baseline cortisol levels for mirror carp, suggesting chronic mild stress resulting from long term exposure to PAA. In a recent study in salmon, a 30-min single exposure and re-exposure to PAA in the concentration of 0.6 and 4.8 mg/L increased plasma cortisol levels (Soleng et al. 2019). In the present study, however, no significant changes in plasma cortisol levels were observed following periodic low-dose PAA exposure. Moreover, observed mean cortisol levels in the present study were in line with reported baseline levels for salmon (Barton and Iwama 1991). The data indicate that the periodic low-dose application of PAA was not a strong enough chemical oxidant stressor to considerably increase plasma cortisol levels.

Plasma glucose levels increase to meet increased energy requirements during stressful events (Mommsen et al. 1999). It is suggested that elevated cortisol levels modulate the production of glucose by enhanced glycogenolysis and gluconeogenesis during stress (Mommsen et al. 1999; Fast et al. 2008). Since no changes in basal cortisol levels were observed in the present study, a significant increase in basal glucose levels was not anticipated. The plasma glucose levels remained unaltered during the first 2 weeks of exposure, indicating that salmon can metabolically cope with low-dose PAA, as with other stress parameters. Such observation supports a previous study (Soleng et al. 2019), where exposure to PAA at distinct concentrations did not alter the glucose levels at 2h, 48h and 2

weeks post-exposure compared to pre-exposure levels. Interestingly, a significant decrease in glucose levels at days 22 and 45 post-exposure relatively to pre-exposure was observed in the present study. This decreasing dynamic suggests a form of adaptation, where fish recognised that periodic PAA exposure did not represent an energy demanding stressor. The measured glucose levels were within baseline values for salmon despite the apparent decrease, thus, supporting this inference (Mommsen et al. 1999).

Lactate levels increase as a result of anaerobic metabolism during stressful periods (Wendelaar Bonga 1997; Sopinka et al. 2016). Soleng et al. (2019) reported that exposure and subsequent re-exposure to PAA did not significantly affect the plasma lactate levels in salmon. In the current study, temporal variations in the levels of lactate were observed, with a transitory increase in lactate levels followed by a tendency of recovery to pre-exposure levels at day 45 of exposure. Therefore, even though PAA administration might have provoked some level of stress, the magnitude of stress did not appear to be strong enough to activate strong responses from the anaerobic metabolism of the fish. Supporting this hypothesis is the fact that no behavioural changes, namely increased swimming activity or agitation, were detected following PAA exposure (Lazado, Sveen, et al. 2020) .

Since PAA is a known source of ROS, PAA-based disinfectants can induce a transitory state of oxidative stress in exposed fish before full decay of components is achieved (Liu et al. 2020). Antioxidants have the ability to scavenge ROS and prevent cellular oxidative stress, hence, elevated plasma TAC indicates mobilisation of antioxidants to counteract redox imbalance following oxidative stress (Soleng et al. 2019). In the present study, TAC levels showed a significant elevation at days 22 and 45 post-exposure compared with pre-exposure levels. This increase suggests that exposure to PAA resulted in internal redox imbalance, thereby eliciting the activation of systemic antioxidants to scavenge excess ROS and maintain redox homeostasis. This elevation is likely a protective mechanism against PAA-induced oxidative stress, as previously suggested (Soleng et al. 2019; Liu et al. 2020).

5.2 Structural and morphometric changes in the mucosal organs of salmon after periodic PAA exposure

No significant pathological changes were identified in the skin in any of the sampling points. However, the witnessed higher scores for epithelial surface quality following periodic PAA exposure are indicative that exposure might have compromised the epithelial surface of the skin to some extent. The observed increase in dermal thickness at 45 days of exposure could act as a compensatory mechanism of the skin, providing additional protection from diffusion/uptake of PAA and H_2O_2 despite the slightly compromised epithelial surface. Another possible explanation is that the observed increase in dermal thickness is a physiological consequence of salmon development (Wilkins and Jancsar 1979). In fact, a significant

correlation was found between dermal thickness and weight ($P<0.001$, $r=0.71$), and length ($P<0.001$, $r=0.71$) of the fish. In general, these results are supported by previous studies where no significant histostructural changes in the skin were observed after different regimens of PAA exposure (Lazado, Haddeland, et al. 2020; Lazado, Sveen, et al. 2020; Liu et al. 2020).

The gills are a multi-purpose organ, responsible for respiration, maintaining optimal osmotic pressure and acid-base balance of body fluids but, because gills are in direct contact with the water, they are particularly vulnerable to a variety of injuries (Wolf et al. 2015; Strzyzewska et al. 2016). The decreased interlamellar space observed at day 45 was most likely the result of hyperplasia, hypertrophy and oedema cases in the base of the lamellae. Although these alterations may act as a protective and adaptive mechanism by augmenting the oxidant diffusion distance, the respiratory surface becomes reduced, as a result, impairing gill function to some extent (Roberts 2012). Increased numbers of mucous cells have been documented in response to persistent gill irritation (Wolf et al. 2015; Strzyzewska et al. 2016). The concurrent increase in the number of acidic mucous cells may reflect a defence mechanism since higher proportions of acidic mucous cells are linked with an increase in the viscosity of mucus that helps to prevent chemical damage to the epithelium (Vatsos et al. 2010).

Currently, conflicting data on gill histopathological alterations due to PAA exposure exist (Straus et al. 2012; Chupani et al. 2014; Chupani et al. 2016; Liu et al. 2018; Liu et al. 2020; Lazado, Sveen, et al. 2020), likely resulting from different parameters between the conducted studies and the composition of PAA trade products (Lazado, Haddeland, et al. 2020). In the present study, even though histopathological lesions became more prevalent and pronounced following PAA exposure, cases of necrosis and lamellar fusion were disperse and only sporadically identified. Moreover, only cases of epithelial lifting, hypertrophy, hyperplasia and lamellar clubbing, which are all reversible lesions (Roberts 2012; Wolf et al. 2015), increased markedly following periodic PAA exposure. Therefore, it seems that periodic oxidant exposure led to some reversible pathological changes in the gills, the overall gill health status was not severely compromised, and if given enough time, the fish would likely show a recovery from the reported lesions.

In salmonids, the olfactory epithelium lines a multi-lamellar olfactory rosette which is covered by sensory epithelium, highly sensitive to water contaminants, and non-sensory epithelium (Hansen and Zielinski 2005; Ghosh and Chakrabarti 2010). The olfactory rosette of rainbow trout has been shown to contain abundant myeloid and lymphoid cells, thus having a strong capacity to mount innate and adaptive immune responses (Tacchi et al. 2014). In the current study, the documented enlargement of the epithelium and lamina propria could act as an enhanced protection barrier against the uptake of oxidant to safeguard the integrity and function of the olfactory organ. Moreover, it could be that given its more external positioning,

the epithelium presents an earlier point of contact than the lamina propria to the oxidant, which could justify the earlier increase of epithelium thickness. A significant increase in the secretion of olfactory mucous is described in response to the presence of various chemical compounds. Thus, the apparent increase in the density of mucous cells at the tip of olfactory lamellae likely provides additional protection of the olfactory sensory neurons and regulation in the detection of external cues and chemosensory responses (Kasumyan 2004).

5.3 Distinct modulation of the expression of stress and immune-related genes in mucosal organs after periodic PAA exposure

In the present study, the observed modulation of gene expression in the mucosal organs was primarily marked by a reduction in transcript levels, indicating that a potential form of adaptation to periodic oxidant exposure took place.

PAA exposure significantly downregulated the expression of *gr* and *cu/znsod* in the gills, a pattern previously described (Chupani et al. 2016; Soleng et al. 2019). *Cu/znsod* catalyses the dismutation of superoxide radicals to H_2O_2 and O_2 to neutralise oxygen radical-mediated toxicity (Chalmers et al. 2018). The presence of H_2O_2 in PAA-based products could somewhat explain the decreased expression of *cu/znsod* in the gills since high levels of H_2O_2 can inhibit SOD activity (Kosenko et al. 1997; Chupani et al. 2016). *Gr* is responsible for the regeneration of reduced glutathione, a crucial step in cellular antioxidant protection (Doyotte et al. 1997). In the skin, upregulation of *gr* expression was observed, an opposite profile compared with the gills. These contradicting patterns of *gr* expression indicate that glutathione-mediated response likely differs between the gills and skin. As for the olfactory rosette, decreasing expression of *cat* was observed. *Cat* catalyses the transformation of H_2O_2 into O_2 and water when present at high concentrations (Solé et al. 2009), and periodic exposure likely hindered this process. The responsiveness of antioxidant genes *gr*, *cu/znsod* and *cat* corroborates their acknowledged role in oxidative stress response (van der Oost et al. 2003; Olsvik et al. 2005), and the changes observed imply their vital function in protecting the mucosa towards PAA-induced oxidative damage. Nevertheless, no drastic and substantial changes occurred in the overall expression profiles of the antioxidant genes in the three mucosal tissues, which suggests that periodic PAA exposure only triggered minimal oxidative stress at the mucosal level.

Hsp70 acts as a molecular chaperone and facilitates the repair and elimination of altered or denatured proteins, whereas *hsp90* has an essential role in supporting various components of the cytoskeleton, enzymes and steroid hormone receptors (Basu et al. 2002; Iwama et al. 2004). A transient decrease of *hsp90* expression was observed in the gills, while in the olfactory rosette, the expression of both *hsp70* and *hsp90* decreased after periodic

exposure to PAA. Two potential explanations can be given as to why the decrease in expression. First, although PAA triggered oxidative stress, it was mild and thus did not elicit countermeasures from *hsps*, which have a known protective role in oxidative damage (Basu et al. 2002), since antioxidants factors may already be at play. On the other hand, the decrease in expression may be related with the diminishing response to repeated encounter to an oxidant, which further suggests a form of desensitisation or habituation.

Interleukins are a subset of cytokine molecules involved in the intercellular regulation of the immune system (Secombes et al. 2011). As earlier discussed, *il1β* is a pro-inflammatory cytokine, whereas *il10* is an anti-inflammatory cytokine (Elenkov and Chrousos 2002; Chalmers et al. 2018), and their expression can be induced by multiple stressors (Ingerslev et al. 2009; Secombes et al. 2011). The reduction in the expression of *il1β* and *il10* observed in the gills is indicative that PAA may interfere with the inflammatory process in response to a strong oxidant by inhibiting two crucial molecular regulators. These cytokines likely played a role in the progression of histopathological lesions documented in the gills throughout the 3 major sampling points.

Mucins are the main components of the mucous and are high molecular weight, filamentous and highly glycosylated glycoproteins, playing a crucial role in mucosal defence (Shephard 1994; Sveen et al. 2017). Periodic exposure to PAA only modulated the transcription of gel-forming mucins (*muc5ac*, *muc5b* and *muc2*) in the gills and skin, where a general profile displayed transient downregulation at mid-exposure, and then returned to the pre-exposure level at the end of exposure. Exposure to PAA had no direct impact on the phenotypic properties of the mucous cells in the skin since their morphometries were unaffected. However, the effects seemed to be more significant on the biochemical level, such as the mucins. Furthermore, the overall profile suggests a potential recovery following a transcriptional reduction midway of the exposure period. Despite a noticeable increase in the mucous cell population of the nasal olfactory mucosa, none of the mucin genes in the study were significantly affected. This contrast indicates that PAA effects in the nasal mucosa were mainly at the phenotypic level, with more mucous cells contributing to improved protection of the mucosa against an irritating oxidant, and not at the biochemical level, which is quite distinct compared with the gill and skin profile. Since mucin is a large class of glycoproteins, there are perhaps other mucins that were differentially affected within the mucosal organs but were not investigated in the present study.

The liver represents an essential organ in xenobiotic metabolism (Roberts 2012), and as such, may play a role in the organisms' responses to oxidants present in the aquatic environment. Nonetheless, no remarkable changes were observed in the expression of selected genes in the liver, suggesting that the impacts of periodic PAA exposure on the

transcriptional profile of stress and immune-related genes occurred predominantly at the mucosal tissues.

5.4 Effects of periodic PAA exposure in the physiological stress responses of Atlantic salmon subjected to handling-confinement stress

Exposure to chemical pollutants or toxicants is known to potentially cause exhaustion of the pituitary-interrenal axis and consequently impair the ability of the fish to increase cortisol levels in response to an acute stressor (Wendelaar Bonga 1997). In contrast, chronic exposure to mild stressors may also desensitise fish and mitigate the neuroendocrine and metabolic responses to acute stressors (Barton 2002). Gesto et al. (2018) reported that both unexposed and PAA-exposed (either periodically or continuously) rainbow trout exhibited an increased plasma cortisol level after chasing stress, revealing that PAA exposure did not change the typical cortisol response of the fish when challenged with a secondary stressor. In the current experiment, the cortisol response following handling and confinement demonstrated a similar pattern of elevated plasmatic levels before and after periodic PAA exposure. Remarkably, the cortisol level at 3h was significantly higher in the group periodically exposed to PAA than the unexposed group. These observations suggest that previous periodic exposure to PAA did not dramatically alter the ability of fish to mount a classical cortisol response to an acute secondary stressor. However, it may have a slight influence on the kinetics of cortisol recovery, which would be interesting to explore in future studies. Both glucose and lactate levels following exposure to handling-confinement stress did not significantly change in the pre-exposed and periodically exposed groups. The data further support the implication that periodic PAA exposure does not alter the physiological responses of salmon to a secondary stressor (Soleng et al. 2019).

5.5 Bacterial activity in the water phase and the biomedia of the MBBR

A requirement for the use of PAA-based products as chemoterapeutants in RAS is that, at the selected concentrations, PAA does not impair the welfare of the treated fish, as well as the nitrification process carried out by the biofilter (Pedersen et al. 2009). Microbial water quality in RAS is critical for the successful operation of these systems, but it remains difficult to evaluate and to maintain (Rojas-Tirado et al. 2017). BactiQuant ® Water has been successfully employed for the fast, reliable, culture-independent determination of bacterial activity in RAS water (Pedersen et al. 2017; Rojas-Tirado et al. 2017), hence allowing to monitor microbial water quality. The relatively increased bacterial activity in the water-phase registered at 1 day before the start of exposure and at day 3 of periodic exposure was likely the result of augmented organic matter content in the water, which in turn resulted from a

period of adaptation of the fish to higher feeding loads. As for the marked reduction of bacterial activity in the water at day 44 of periodic exposure, it can be explained by the fact that the recirculation system was running at a higher water flow rate in the previous day, which led to higher replacement of water and less accumulation of organic content. Despite some temporal fluctuations in the bacterial activity in the water, which are common in the starting weeks of RAS production (Rojas-Tirado et al. 2017), bacterial activity remained fairly stable after the start of PAA exposure. Periodic applications of PAA at 1 mg/L did not seem to impair the microbial activity of the system.

In addition, water samples were also collected during the trial for the quantification of total ammonia nitrogen, nitrite and nitrate levels, which remained within acceptable range. However, the data are not included in the current thesis and are instead included in the thesis of another student also affiliated with the trial.

The existence of a balanced microorganism population is required in RAS, as it is crucial for maintaining adequate water quality. Since RAS are intensive aquaculture systems that function with high rates of water reutilization, these systems depend on the constant and effective removal of ammonium and nitrite through the process of biological filtration (Pedersen et al. 2009). Application of PAA at 1 mg/L was reported to partially impair the nitrification process in different production environments (Pedersen et al. 2009; Liu, Straus et al. 2017). In the present study, increased bacterial activity was identified in the surface of the biofilter media at days 21 and 44 of PAA exposure relative to 5 days before the beginning of exposure. Such increase in bacterial activity is most likely due to higher water flow rates in the system at days 20 and 43 of periodic exposure, which favoured bacterial growth in the biofilter compartment. However, no significant changes were observed in the bacterial activity in the surface of the biofilter media from the start to end of PAA exposure, indicating that periodic disinfection with PAA did not negatively affect the bacterial population of the biofilter. It was not anticipated that the selected protocol of disinfection with PAA at 1 mg/L would affect the stability and performance of the biofilter, since the experiment was conducted in brackish water RAS tanks with high organic matter content, which promotes the rapid degradation of PAA (Pedersen et al. 2009; Liu et al. 2014; Pedersen and Lazado 2020).

6 Conclusions

The present study revealed that periodic exposure of salmon to PAA, a strong oxidant, initiated physiological and histostructural changes, underscoring both mucosal and systemic adaptations. No drastic changes were observed in the plasmatic levels of the classical stress indicators, implying that PAA provoked low levels of systemic stress. PAA seemed to cause an internal redox imbalance leading to systemic oxidative stress, which was compensated with increased production of systemic antioxidants. Periodic PAA exposure differentially affected several genes encoding for antioxidants, cytokines, heat shock proteins and mucins - where downregulation was the prominent profile in the gills and olfactory rosette, whereas upregulation was present in the skin. Such a distinct profile reveals that mucosal organs react differently to PAA, which may be important in coordinating a robust mucosal response to the oxidant. While PAA led to varying levels of histostructural alterations in the three mucosal organs, the gills were substantially affected, with reversible pathological lesions becoming more frequent following periodic oxidant exposure. PAA did not affect the ability of salmon to assemble a physiological stress response in the presence of a secondary stressor, indicating that PAA is a welfare-friendly antimicrobial oxidant.

The data presented here underline the biological consequences of a particular PAA-based disinfectant (Perfectoxid) in post-smolt salmon reared in brackish water RAS, where the overall profile demonstrates that even though it is a potential mild stressor that poses strong physiological demands, the fish were able to coordinate an interconnected response likely in the form of adaptation and habituation to its presence in the rearing environment. Moreover, PAA administrations contributed to the maintenance of stable microbial water quality without affecting the biofilter performance. These results further support the potential application of PAA as a routine disinfectant in salmon RAS production.

7 Recommendations

The following recommendations should be considered for future studies:

1. Since PAA is commercially available under different strengths and stabilizers, it would be interesting to compare different trade products to investigate whether different product formulations will influence the physiological responses of salmon to PAA exposure;
2. The current study was only conducted for a period of 6 weeks, and therefore there is a need to assess the impacts of longer periods of PAA exposure;
3. As salmon goes through several production stages, future studies should investigate the long-term impacts of PAA, namely the impacts of PAA applications during land-based production once the fish are transferred to sea cages;

4. The interaction of different environmental factors such as water temperature, density, and organic matter content on the responses of salmon to PAA should be investigated;
5. Future studies should likewise test whether the present protocol of disinfection is effective in the control of pathogens in case a break in biosecurity occurs, for example if a certain pathogen enters the system.

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Appendix I – Protocol for the determination of plasma cortisol concentration

1. For each run, microplate wells were prepared to accommodate calibrators, controls and samples in duplicates.
2. 10 µL of each calibrator (0, 10, 30, 90, 270 and 800 ng/mL), sample and control were added in duplicate into the wells.
3. 200 µL of Enzyme Conjugate were added into each well.
4. Microplate was covered by adhesive film and incubated for 60 minutes at room temperature (22ºC) and 700 rpm using a ThermoMixerC plate shaker (Eppendorf AG, Hamburg, Germany). To achieve complete mixing, the microplate was thoroughly mixed for 10 seconds before incubation.
5. Content of the wells was discarded, and the wells were rinsed 4 times with 300µL of 1:10 diluted Wash Solution. The microplate was then beaten on absorbent lint-free paper to remove as much Wash Solution as possible.
6. 200 µL of Substrate Solution were added to each well, and the microplate was incubated without shaking for 30 minutes in the dark.
7. Reaction was stopped by adding 50 µL of Stop Solution to each well.
8. Optical density (OD) of each well was determined at 450 nm using a SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenberg, Germany).

For the calculation of plasma cortisol concentrations (ng/mL), the duplicate OD readings for each standard and sample were averaged. A standard curve was then created by reducing the data and the sample concentrations extrapolated, using a 4 Parameters Logistics Curve Fit in Microsoft Office Excel 2013 © software.

Appendix II – Protocol for the determination of plasma glucose concentration

Before the start of the test, plasma samples were diluted 1:30 with deionized water. HRP stock solution and GOD solution were diluted 1:100 and 1:10, respectively, with Assay Buffer. Glucose Standards were prepared following the manufacturer's protocol:

1. 1.5 mL Eppendorf tubes were labelled as #1 through #7.
2. Glucose Standard was briefly vortexed to ensure proper mixing.
3. 135 µL of Assay Buffer were pipetted into tube #1.
4. 75 µL of Assay Buffer were pipetted into tube #2 to #7.
5. 15 µL of Glucose Standard were added into tube #1 and tube was thoroughly vortexed.
6. 75 µL of the solution in tube #1 were added into tube #2 and thoroughly vortexed.
7. The previous step was repeated for tubes #3 through #7.

The colorimetric glucose test was performed as follows:

1. For each run, a sufficient number of microplate wells was prepared to accommodate standards and samples in duplicates.
2. 20 µL of diluted Samples and Standards (32, 16, 8, 4, 2, 1 and 0.5 mg/dL) were added into duplicate wells in the plate.
3. 20 µL of Assay Buffer were added into duplicate wells as the Zero Standard.
4. 25 µL of the diluted HRP solution were added to each well.
5. 25 µL of the diluted Substrate solution were added to each well using a repeater pipette.
6. 25 µL of the prepared GOD solution were added to each well using a repeater pipette to initiate the reaction.
7. Plate was incubated at room temperature for 30 minutes.
8. Optical Density of each well was determined at 560nm using a SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenberg, Germany).

To calculate plasma glucose concentrations (mg/dL), the duplicate OD readings for each standard and sample were averaged. After subtracting the mean ODs for the Zero Standard wells, a standard curve was created by reducing the data, using a 4 Parameters Logistics Curve Fit in Microsoft Office Excel 2013 © software. The sample concentrations obtained were then multiplied by the dilution factor to obtain actual sample concentrations.

Appendix III – Isolation of ribonucleic acid

(1) Sample Lysis/Homogenization

1. Approximately 20 mg of tissue sample was cut and transferred to 1.5 mL crushing tubes containing 350 µL of RNA Lysis Buffer.
2. Tissue samples were homogenized for 5 minutes at room temperature and 1800 rpm using the fast prep 96^{TM} plate shaker (MP Biomedicals, Ohio, USA).

(2) Sample Clearing:

1. Samples were centrifuged for 1 minute and 1800 rpm using Avanti J-30I (Beckman Coulter, Indianapolis, USA) and supernatant was transferred to a RNase-free tube.

(3) RNA Purification:

1. 350 µL of ethanol were added to the sample in RNA Lysis Buffer and the obtained solution was thoroughly mixed.
2. Mixture was transferred to a Zymo-Spin^{TM} IC Column in a Collection Tube and centrifuged for 30 seconds at 22ºC and 14000 rpm (5424 R, Eppendorf AG, Hamburg, Germany). Flow-through was discarded.
3. For the removal of trace DNA, column was prewashed with 400 µL RNA Wash Buffer, centrifuged for 30 seconds and flow-through was discarded. 40 µL of DNase I Reaction Mix were directly added to the column matrix and samples incubated at room temperature for 15 minutes.
4. 400 µL of RNA Prep Buffer were added to the column and centrifuged for 30 seconds. Flow through was discarded.
5. 700 µL of RNA Wash Buffer were added to the column and centrifuged for 30 seconds. Flow through was discarded.
6. 400 µL of RNA Wash Buffer were added to the column and centrifuged for 2 minutes to guarantee complete removal of the wash buffer. The column was then transferred into a RNase-free tube.
7. 15 µL of DNase/RNase-Free Water were added to the column matrix and centrifuged for 30 seconds.
8. The eluted RNA samples were stored at -70ºC until further use.

Appendix IV - Determination of bacterial activity in the water phase

1. Water samples were collected from the four tanks using a 60 mL syringe.
2. Calibration of the fluorometer was performed using a standard solution.
3. A blank value was determined by measuring the fluorescence of a developer and substrate mix, to verify good conservation of the substrate.
4. Excess water was flushed from the sampling syringes until reaching a 50 mL volume and sampling syringes were then attached to sterile 0.2 µm filters.
5. Water samples were pushed through the filters into a proper container and syringes were discarded.
6. 2.5 mL of substrate were transferred into 2.5 mL syringes (Terumo, Leuven, Belgium) and the syringes attached to the filters.
7. Developers were transferred into corresponding cuvettes.
8. Filters were flushed with substrate, attached with the syringes to a blunt needle, and left for a reaction time of 30 minutes.
9. After 30 minutes, the filter with the syringe and blunt needle of each sample was placed in the corresponding developer and back flushing two times.
10. Syringes were detached from the filters, refilled with air, and reattached to the syringes, and all remaining liquid in the filter was pushed out to the cuvette.
11. The cuvettes were placed in the fluorometer and read.
12. The obtained analysis values reflected the bacterial activity of the samples, expressed in standardized Bactiquant® Values (BQV).

The following picture shows the layout of the materials used to perform the Bactiquant water analysis.

Appendix V – Determination of the bacterial activity in the media/surface of the Moving Bed Biofilter Reactor

1. Samples were collected from each of the 5 media using a sterile cotton swab wetted in sterile saline solution. The swab was thoroughly rubbed in 5 different directions, both in the dorsal and ventral surfaces of each biofilter media, rotating the swab to use as much area of cotton as possible.
2. Following sample collection, each swab was returned into a proper container until analysis.
3. Calibration of the fluorometer was performed using a standard solution.
4. For each sample, a developer was emptied to a cuvette and read in the fluorometer to obtain the Blank Value 1 (BV1).
5. 100 µL of substrate were transferred to the developer in each cuvette. Cuvettes were read to obtain Blank Value 2 (BV2).
6. A reaction time was set according to the temperature in the lab.
7. Swabs were transferred to the substrate tubes and mashed to ensure proper mixing.
8. Timer was started and the Combined Blank Value (CV) was calculated for each sample using the formula: CV= BV2 + (BV2-BV1).
9. At the end of reaction time, 100 µL of Substrate solution sample were transferred to the corresponding cuvette and thoroughly mixed using the pipette tip.
10. Cuvettes were read and the Analysis Value (AV) was recorded for each sample.
11. A Bactiquant® Value (BQV) was calculated for each sample using the following formula: BQV = AV – CV.